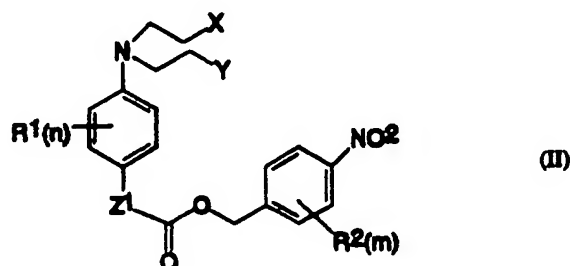
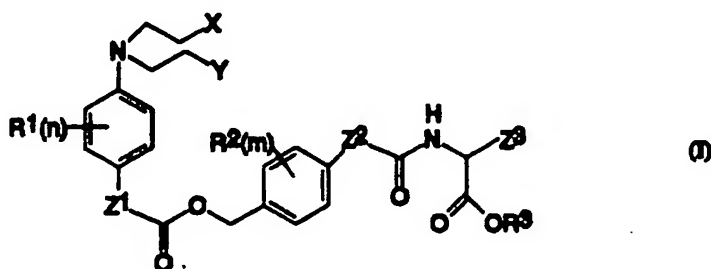


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(21) International Application Number: PCT/GB96/00112 (22) International Filing Date: 19 January 1996 (19.01.96) (30) Priority Data: 9501052.6 19 January 1995 (19.01.95) GB (71) Applicant (for all designated States except US): CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED (GB/GB); 6-10 Cambridge Terrace, Regent's Park, London NW1 4JL (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): SPRINGER, Caroline, Joy (GB/GB); The Institute of Cancer Research, The Royal Marsden Hospital, 15 Cotswold Road, Belmont, Sutton, Surrey SM2 2NG (GB). NICULESCU-DUVAZ, Ion (RO/GB); The Institute of Cancer Research, The Royal Marsden Hospital, 15 Cotswold Road, Belmont, Sutton, Surrey SM2 2NG (GB). (74) Agents: BRASNETT, Adrian, Hugh et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>

(54) Title: **NITROGEN MUSTARD PRODRUGS WITH NOVEL LIPOPHILIC PROTECTING GROUPS, AND PROCESSES FOR THEIR PRODUCTION**



(57) Abstract

The invention provides compounds of formula (I) and (II) wherein X and Y are independently chlorine, bromine, iodine, a mesyl group CH_3SO_2 or a tosyl group $\text{OSO}_2\text{phenyl}$ (wherein phenyl is optionally substituted by 1, 2, 3, 4 or 5 substituents independently selected from C_1 -alkyl, halogen, cyano or nitro; R^1 and R^2 are independently 1 to 4 optional substituents; Z^1 and Z^2 are each independently -O- or -NH-; R^3 is hydrogen, *t*-butyl or allyl; Z^3 is a hydrocarbyl group such as carboxyethyl, optionally containing heteroatoms, and physiologically acceptable derivatives thereof. The compounds can be converted *in situ* into nitrogen mustard agents by the actions of enzymes such as carboxypeptidase or nitroreductase and are useful for the treatment of cancer.

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NITROGEN MUSTARD PRODRUGS WITH NOVEL LIPOPHILIC PROTECTING GROUPS, AND PROCESSES FOR THEIR PRODUCTION

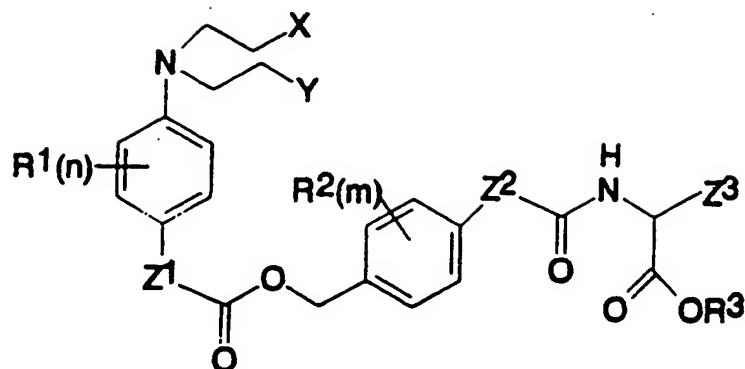
The present invention relates to prodrugs and their use in the treatment of disease, including cancers.

5 The use of prodrugs represents a clinically very valuable concept in cancer therapy since, particularly where the prodrug is to be converted to an anti-tumour agent under the influence of an enzyme that is linkable to a monoclonal antibody that will bind to a tumour associated antigen, the combination of such a prodrug with such an enzyme monoclonal/antibody conjugate represents a very powerful clinical agent. This approach to cancer therapy, often referred to as "antibody directed enzyme/prodrug therapy" (ADEPT) is disclosed in WO88/07378.

10 More recently, a similar approach ("GDEPT", Gene-Directed Enzyme Prodrug Therapy) has been proposed where in place of an antibody/enzyme conjugate, tumour cells are targeted with a viral vector carrying a gene encoding an enzyme capable of activating a prodrug. The gene may be transcriptionally regulated by tumour specific promoter or enhancer sequences. The viral vector enters tumour cells and expresses the enzyme, in order that a prodrug is
15 converted to an active drug only in the vicinity of the tumour cells (Huber et al. Proc. Natl. Acad. Sci. USA (1991) 88, 8039). The enzyme may be targeted to particular subcellular locations or expressed at the cell surface.

The present invention provides novel prodrugs which contain protecting groups which are more lipophilic than compounds of the prior art such as those disclosed in WO88/07378.

20 Accordingly, the present invention provides compounds of the formula (I):



wherein X and Y are independently chlorine, bromine, iodine, a mesyl group CH_3SO_2 or a tosyl group $\text{OSO}_2\text{phenyl}$ (wherein phenyl is optionally substituted by 1, 2, 3, 4 or 5 substituents independently selected from C_{1-4} alkyl, halogen, cyano or nitro:

n is an integer from 0 to 4 and each group R^1 , which may be the same or different when n is greater than 1, is fluorine, chlorine, bromine, iodine, hydroxy, mercapto, amino, nitro, C_{1-4} alkyl optionally containing one double bond or one triple bond, C_{1-4} alkoxy, $-\text{CONR}^7\text{R}^8$ (wherein R^7 and R^8 are as defined below), $-\text{NH}(\text{C}_{1-4}\text{-alkyl})$, $-\text{N}(\text{C}_{1-4}\text{-alkyl})_2$ or $\text{C}_{2,3}$ alkanoyl; or when n is 2 or more and two groups R^1 are on adjacent positions on the phenylene ring, 2 adjacent R^1 groups together represent

- 10 a) C_4 alkylene optionally having one double bond;
- b) C_3 alkylene; or
- c) $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-$, $-\text{CH}=\text{CH}-\text{CH}_2-$ or $-\text{CH}_2-\text{CH}=\text{CH}-$ each optionally substituted with 1, 2, 3 or 4 substituents said substituents each independently selected from the group consisting of C_{1-4} alkyl, C_{1-4} alkoxy, halogen, cyano, nitro, $\text{C}_{2,3}$ alkanoyl and $-\text{CONR}^7\text{R}^8$
- 15 (wherein R^7 and R^8 are as defined below):

m is an integer from 0 to 4 and R^2 , which may be the same or different when m is greater than 1, is fluorine, chlorine, bromine, iodine, hydroxy, mercapto, amino, nitro, C_{1-4} alkyl optionally containing one double bond or one triple bond, C_{1-4} alkoxy, $-\text{CONR}^7\text{R}^8$ (wherein R^7 and R^8 are as defined below), $-\text{NH}(\text{C}_{1-4}\text{-alkyl})$, $-\text{N}(\text{C}_{1-4}\text{-alkyl})_2$ and $\text{C}_{2,3}$ alkanoyl; or when

20 n is 2 or more and two groups R^1 are on adjacent positions on the phenylene ring, 2 adjacent R^1 groups together represent

- a) C_4 alkylene optionally having one double bond;
- b) C_3 alkylene; or

c) $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-$, $-\text{CH}=\text{CH}-\text{CH}_2-$ or $-\text{CH}_2-\text{CH}=\text{CH}-$ each optionally substituted with 1, 2, 3 or 4 substituents said substituents each independently selected from the group consisting of C_{1-4} alkyl, C_{1-4} alkoxy, halogen, cyano, nitro, C_{2-5} alkanoyl and $-\text{CONR}_7\text{R}_8$ (wherein R_7 and R_8 are as defined below);

5 Z^1 and Z^2 are each independently $-\text{O}-$ or $-\text{NH}-$;

R^3 is hydrogen, *t*-butyl or allyl;

Z^3 is a group $-\text{V}-\text{W}$ where V is $-\text{CH}_2-\text{T}-$ in which T is $-\text{CH}_2-$, $-\text{O}-$, $-\text{S}-$, $-(\text{SO})-$ or $-(\text{SO}_2)-$ (provided that when V has sulphur or oxygen as its second atom, W is other than $-\text{COOH}$) and said group V optionally further carrying one or two substituents Q_1 and/or Q_2 on carbon;

10 wherein Q^1 and Q^2 each independently represents C_{1-4} alkyl or halogen; or, when Q_1 and Q_2 are bonded to adjacent carbon atoms, Q^1 and Q^2 together may additionally represent a C_3-C_4 alkylene radical optionally substituted with 1, 2, 3 or 4 substituents independently selected from the group consisting of C_{1-4} alkyl and halogen; and W represents

15 (1) COOH ,

(2) $-(\text{C}=\text{O})-\text{O}-\text{R}_6$ wherein R_6 represents a C_{1-6} alkyl, C_{3-6} cycloalkyl or aryl (as defined in 3 below) group;

(3) $-(\text{C}=\text{O})-\text{NR}_7\text{R}_8$ wherein R_7 and R_8 each independently represent hydrogen or a C_1-6 alkyl, C_3-6 cycloalkyl, aryl, heteroaryl linked to N via carbon or C_7-9 aralkyl group wherein

20 aryl is phenyl;

heteroaryl is a 5 or 6 membered ring containing 1 to 3 heteroatoms selected from the group consisting of nitrogen and sulphur;

the aryl moiety *per se*, the heteroaryl moiety and the aryl moiety of the aralkyl group may be substituted on carbon with 1-4 substituents selected from the group consisting of $-\text{COOH}$, $-\text{OH}$, $-\text{NH}_2$, $-\text{CH}_2-\text{NH}_2$, $-(\text{CH}_2)_{1-4}-\text{COOH}$, tetrazol-5-yl and $-\text{SO}_3\text{H}$ and the alkyl moiety may optionally carry a methyl group;

25 (4) $-\text{SO}_2\text{NHR}_9$ wherein R_9 is as defined for R_7 but may additionally represent $-\text{CF}_3$, $-\text{CH}_2\text{CF}_3$ or aryl as defined above;

30 (5) SO_3R_{10} in which R_{10} represents H , C_{1-6} alkyl or C_{3-6} cycloalkyl,

(6) $\text{PO}_3\text{R}_{10}\text{R}_{10}$ (wherein the R_{10} radicals, which may be the same or different, are as herein defined)

(7) a tetrazol-5-yl group;

- (8) -CONH-SO₂R₁₁ in which R₁₁ represents
- (a) C₃₋₇ cycloalkyl;
 - (b) C₁₋₆-alkyl optionally substituted with substituents selected from the group consisting of aryl as defined below, C₁₋₄-alkyl, CF₃ or halogen; and
 - (c) perfluoro-C₁₋₆ alkyl; wherein
aryl is phenyl or phenyl having 1-5 substituents wherein the substituents are selected from the group consisting of halogen, -NO₂, -CF₃, C₁₋₄ alkyl, C₁₋₄ alkoxy, -NH₂, -NHCOCH₃, -CONH₂, -OCH₂COOH, -NH(C₁₋₄-alkyl), -N(C₁₋₄-alkyl)₂, -NHCOOC₁₋₄ alkyl, -OH, -COOH, -CN and -COOC₁₋₄ alkyl; and
- 10 (9) -M-Het wherein M represents S, SO or SO₂ and Het represents a 5 or 6 membered heterocyclic aromatic ring linked to M via a carbon atom of the aromatic ring, said aromatic ring containing 1, 2, 3 or 4 heteroatoms selected from the group consisting of O, N and S said aromatic ring optionally being substituted on carbon atoms of the ring with 1, 2, 3 or 4 substituents selected from the group consisting of -OH, -SH, -
- 15 CN, -CF₃, NH₂ and halogen;
- and physiologically acceptable derivatives of the compound of formula (I).

These prodrugs can be activated at the site of a tumour by a carboxypeptidase enzyme, for example, CPG2 as disclosed in WO88/07378 or WO94/02450. The carboxypeptidase enzyme may be delivered to the site of a tumour by a GDEPT system as described in the prior art.

20 A carboxypeptidase enzyme is capable of removing the glutamic acid or glutamic acid-derivative moiety (which are preferably in the L-configuration) of compounds of the formula (I). While not wishing to be bound by any one particular theory, it is believed the removal of the glutamic acid moiety from the compounds produces an unstable intermediate compound which undergoes self-immolation to remove the 4-amino or 4-hydroxyl-benzylic system

25 (situated between the groups Z¹ and Z²) thus leading to the release of the alkylating nitrogen mustard drug.

CPG enzyme is available from Sigma Chemical Company, Fancy Road, Poole, Dorset, U.K. CPG enzyme was described in: Goldman, P. and Levy, C.C., PNAS USA, 58: 1299-1306 (1967) and in: Levy, C.C. and Goldman P., J. Biol. Chem., 242: 2933-2938 (1967).

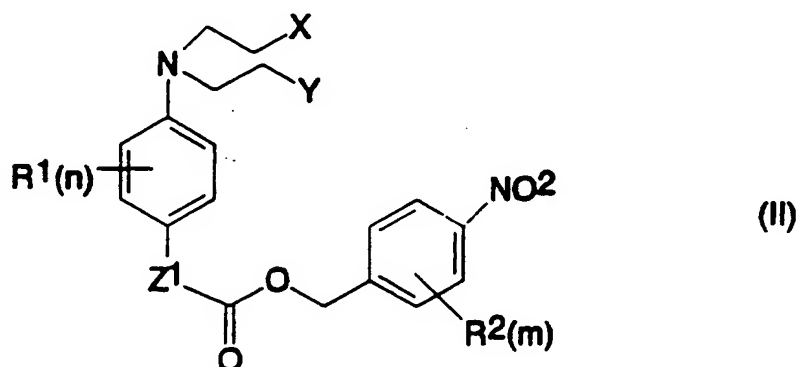
30 Carboxypeptidase G3 enzyme has been described in Yasuda, N. *et al.*, Biosci. Biotech.

Biochem., 56: 1536-1540 (1992). Carboxypeptidase G2 enzyme has also been described in European Patent 121 352.

Large scale purification of CPG2 from Pseudomonas RS-16 was described in Sherwood *et al.* (1985), Eur. J. Biochem., 148, 447-453.

CPG2 may also be obtained by recombinant techniques. The nucleotide coding sequence for CPG2 has been published by Minton, N.P. *et al.*, Gene, 31 (1984), 31-38. Expression of the coding sequence has been reported in *E.coli* (Chambers, S.P. *et al.*, Appl. Microbiol. Biotechnol. (1988), 29, 572-578) and in Saccharomyces cerevisiae (Clarke, L. E. *et al.*, J. Gen Microbiol, (1985) 131, 897-904). Total gene synthesis has been described by M. Edwards in Am. Biotech. Lab (1987), 5, 38-44.

The present invention further provides nitrogen mustard prodrugs of the formula (II):



wherein X, Y, R¹, n, Z, R² and m are as defined for compounds of the formula (I), and physiologically acceptable derivatives thereof.

These prodrugs can be activated at the site of a tumour by a nitroreductase enzyme, for example, as disclosed in WO93/08288. The nitroreductase enzyme may be delivered to the site of a tumour by a GDEPT system as described in the prior art.

Physiologically acceptable derivatives of said prodrugs of formulae (I) and (II) include salts, esters and salts of esters. Esters include carboxylic acid esters in which the non-carbonyl moiety of the ester grouping is selected from straight or branched chain C₁₋₆alkyl, (methyl,

n-propyl, n-butyl or t-butyl); or C_{3-6} cyclic alkyl (e.g. cyclohexyl). Salts include physiologically acceptable base salts, eg derived from an appropriate base, such as alkali metal (e.g. sodium), alkaline earth metal (e.g. magnesium) salts, ammonium and NR_4 (wherein R is C_{1-4} alkyl) salts. Other salts include acid addition salts, including the hydrochloride and acetate salts.

Preferred values of X and Y are independently chloro, iodo and mesyl.

Preferably, n either is zero or is 1 or 2 and the group(s) R^1 are independently fluorine, chlorine, bromine, iodine or C_{1-4} alkyl. Similarly, it is preferred that either m is zero or is 1 or 2 and the group(s) R^2 are independently fluorine, chlorine, bromine, iodine or C_{1-4} alkyl.

10 R^3 is preferably hydrogen.

Z^3 is preferably a group -V-W where V is $-CH_2CH_2-$ and W is a group $-COOH$, $-(C=O)-OR^6$ where R^6 is as defined above, or $-(C=O)-NR^7R^8$ where R^7 and R^8 are independently hydrogen or C_{1-6} alkyl.

15 Preferred compounds of the formula (I) are those in which Z^1 , Z^2 , n and m are as defined above, and:

X and Y are independently selected from chloro, iodo or mesyl;

each phenylene group which may be optionally substituted by R^1 and R^2 is unsubstituted or if either is substituted, the group(s) R^1 and/or R^2 are independently selected from fluorine, chlorine, bromine, iodine or C_{1-4} alkyl;

20 R^3 is hydrogen; and

Z^3 is a group $-CH_2CH_2-COOH$.

Compounds of the formula (I) which are especially preferred are those in which X and Y independently represent iodo or chloro; n and m are both zero;

25 R_1 represents hydrogen; Z_1 and Z_2 each independently represent $-NH-$ or $-O-$; and Z_3 represents CH_2CH_2COOH .

Preferred compounds of the formula (II) are those in which Z¹ is -O- or -NH-. X and Y are
The independently selected from iodo and mesyl, each phenylene group which may be
optionally substituted by R¹ and R² is unsubstituted or if either is substituted, the group(s) R¹
and/or R² are independently selected from fluorine, chlorine, bromine, iodine or C₁₋₄ alkyl,
5 most preferably fluorine, and in which case n and/or m are 1 or 2.

Compounds of the formula (II) which are especially preferred are those in which X represents
iodo; n represents zero; Z¹ represents -O- or -NH-; R₂ represents hydrogen or fluorine; and
m represents an integer from 1 or 2.

The invention further provides pharmaceutical formulations. Such formulations comprise a
10 compound of formula (I) or (II) together with one or more pharmaceutically acceptable
carriers or diluents.

Pharmaceutically acceptable carriers or diluents include those used in formulations suitable
for oral or parenteral (e.g. intramuscular or intravenous) administration. The formulations
may conveniently be presented in unit dosage form and may be prepared by any of the
15 methods well known in the art of pharmacy. Such methods include the step of bringing into
association the active ingredient with the carrier which constitutes one or more accessory
ingredients. In general the formulations are prepared by uniformly and intimately bringing
into association the active ingredient with liquid carriers or finely divided solid carriers or
both, and then, if necessary, shaping the product.

20 For example, formulations suitable for parenteral administration include aqueous and non-
aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and
solutes which render the formulation isotonic with the blood of the intended recipient; and
aqueous and non-aqueous sterile suspensions which may include suspending agents and
thickening agents, and liposomes or other microparticulate systems which are designed to
25 target the polypeptide to blood components or one or more organs.

Suitable liposomes include, for example, those comprising the positively charged lipid (N[1-(2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium (DOTMA), those comprising dioleoyl-

phosphatidylethanolamine (DOPE), and those comprising 3β [N-(n',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol).

In GDEPT the prodrug will usually be administered following the administration of a delivery system containing the gene encoding the enzyme. This includes administration of a modified virus encoding an enzyme. Typically, the virus will be administered to the patient and then the uptake of the virus by infected cells monitored, for example by recovery and analysis of a biopsy sample of targeted tissue.

A wide range of viral delivery systems for gene therapy are known in the art. For example EP-A-415 731 describes molecular chimeras comprising a promoter which may be activated in a tumour cell is operably linked to a heterologous gene encoding an enzyme capable of converting a prodrug into a cytotoxic agent. Such molecular chimeras may be used to express carboxypeptidase or nitroreductase in tumour cells, in order to activate prodrugs of the invention. EP-A-415 731 describes incorporation of such molecular chimeras into viral vectors, e.g. adenoviral or retroviral vectors. Such viral vectors may also be adapted for utilization in the present invention.

Other recombinant viral vector delivery systems are described in WO91/02805, WO92/14829, WO93/10814, WO94/21792, WO95/07994 and WO95/14091 the disclosures of which are incorporated herein by reference. Methods for producing vector delivery systems based on the above-mentioned disclosures may be used to deliver vectors encoding nitroreductase or carboxypeptidase to target cells.

The exact dosage regime for GDEPT will, of course, need to be determined by individual clinicians for individual patients and this, in turn, will be controlled by the exact nature of the prodrug and the cytotoxic agent to be released from the prodrug but some general guidance can be given. Chemotherapy of this type will normally involve parenteral administration of both the prodrug and the enzyme or a vector encoding the enzyme and administration by the intravenous route is frequently found to be the most practical.

In GDEPT systems, the amount of vector delivered will be such as to provide an effective cellular concentration of enzyme so that the prodrug may be activated in sufficient

concentrations at the site of a tumour to achieve a therapeutic effect, e.g. reduction in the tumour size. This may be determined by clinical trials which involve administering a range of trial doses to a patient and measuring the degree of infection or transfection of a target cell or tumour. The amount of prodrug required will be similar to or greater than that for ADEPT systems of the type mentioned above. For example, suitable dose of prodrugs in such systems are described in Bagshawe *et al*, 1991 in Antibody, Immunoconjugates and Radio-Pharmaceuticals I, 915-922. Such doses eg. from 5 to 2000 mg/m² (eg about 200 mg/m² per dose (eg every one or two days or weekly) of prodrugs of the invention may be used, although doses outside this range may be used at the discretion of the physician.

- 10 The present invention also provides a system for use in the control of neoplasia in a human or animal subject comprising a vector or other delivery system capable of selectively infecting tumour cells in said subject, said vector carrying a DNA or RNA sequence encoding an enzyme, in association with a prodrug of formula (I) or (II) capable of being converted to a nitrogen mustard compound by the action of said enzyme.
- 15 The present invention extends to a method of treating neoplasia in a human or animal subject requiring such treatment which comprises administering to the host an effective amount of a prodrug of the invention and a modified virus, said modified virus capable of selectively infecting tumour cells in said subject, said virus carrying a DNA or RNA sequence encoding an enzyme capable of converting said prodrug to an active nitrogen mustard compound.
- 20 The present invention also extends to a method of treating neoplasia in a human or animal subject requiring such treatment which comprises administering to the host an effective amount of a prodrug of the invention and a non viral vector system, said non-viral vector system capable of being selectively introduced into tumour cells in said subject, said vector system carrying a DNA or RNA sequence encoding an enzyme capable of converting said
- 25 prodrug to an active nitrogen mustard operably linked to a promoter effective in expressing said enzyme in said cells.

Vectors capable of selectively infecting tumour cells are known in the art. By "selectively infecting" it is meant that the vector, for example a modified virus, will primarily infect tumour cells and that the proportion of non-tumour cells infected is such that the damage to

non-tumour cells by administration of the prodrug will be acceptably low, given the nature of the disease being treated. Ultimately, this will be determined by the physician.

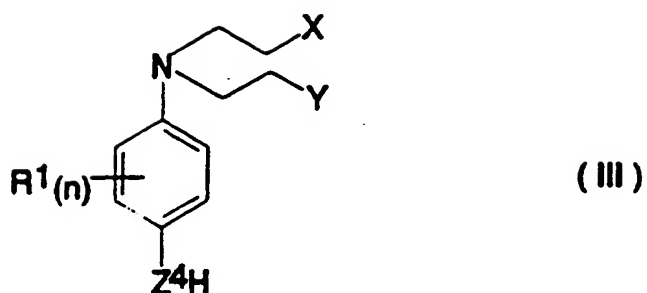
5 It will also be understood that the DNA or RNA sequence encoding an enzyme carried by the virus will be linked to suitable expression control signals such that expression of the enzyme will occur in the targeted tumour cells.

Non-viral vector systems will be capable of being selectively introduced into tumour cells utilizing methods such as those mentioned above, e.g. calcium phosphate co-precipitation, microinjection, liposomes, direct DNA uptake, and receptor-mediated DNA transfer (Morgan & French Anderson, *Annu. Rev. Biochem.*, 1993, 62;191).

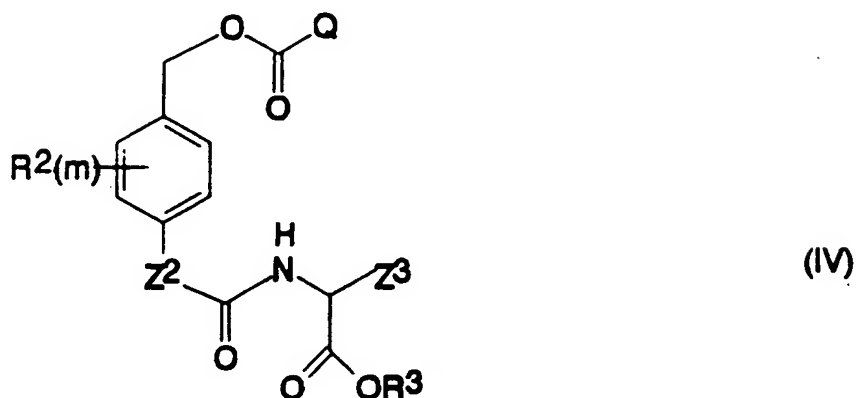
10 Prodrugs of the present invention may also be used as reagents in *in vitro* systems to test the activity of candidate enzymes which may be incorporated into GDEPT systems.

For example, a tumour cell may be infected or transfected *in vitro* by a vector containing an enzyme. The enzyme will be one which is, or suspected to be, capable of converting a prodrug of the invention into an active drug. The prodrug is then added to the culture and
15 the amount of cell killing or inhibition of cell growth is measured (for example by using a vital stain to record the number of viable cells or by replating a sample of the culture to count the number of viable cells). This model system may also be used to test the toxicity, activity or selectivity of compounds of the present invention against a range of tumour cell types suitable tumour cells for use in these model systems include ovarian, colon, glioma, breast,
20 small cell and non-small cell lung cancer cells, and melanomas.

The invention further provides processes for the production of compounds of formula (I) and (II). Thus the invention provides a process for the production of a compound of formula (I) as defined above which comprises reacting a compound of the formula (III)

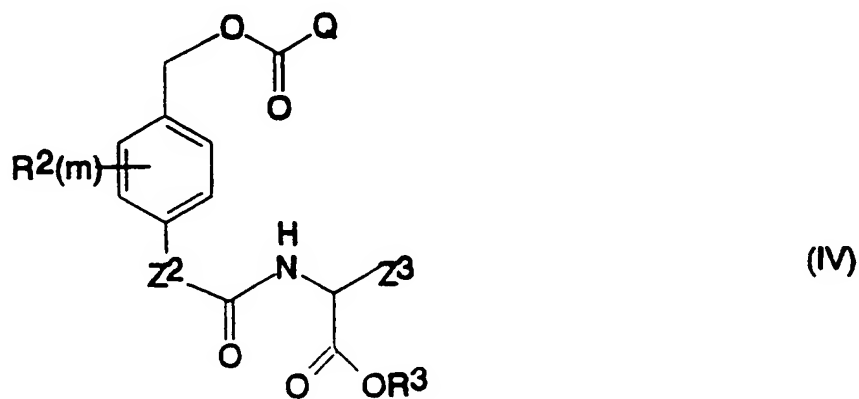


in which X, Y, R¹ and n are as defined for formula (I) and Z⁴ is -O- or -NH- with a compound of formula (IV)



in which R², m, Z², Z³ and R³ are as defined above, and Q is a leaving group. The reaction may be performed in an aprotic solvent and in the presence of a base. The invention also provides a process for the production of compounds of the formula (II) which comprises
 5 reacting a compound of formula (III) as defined above with a 4-nitrobenzyl chloroformate optionally substituted with from 1 to 4 group(s) R² as defined above.

The invention further provides compounds of the formula (IV);



in which R^2 , m , Z^2 , R^3 and Z^3 are as defined above for compounds of the formula (I), and Q is a leaving group, with the proviso that when R^2 is fluorine, chlorine, bromine, iodine, hydroxy, mercapto, amino, nitro, C_{1-4} alkyl, C_{1-4} haloalkyl, C_{1-4} alkoxy, C_{1-4} haloalkoxy, C_{2-4} alkenyl, C_{2-4} alkynyl, and C_{2-4} haloalkenyl, R^3 is hydrogen or a C_{1-6} alkyl group, and Z^3 is a group $-CH_2CH_2COOX^{10}$ where X^{10} is hydrogen or a C1-6alkyl group, then Q is not a N-succinimidyl group. These compounds are useful as linkers which may be attached to active cytotoxic compounds to provide prodrugs of said compounds with increased lipophilicity. The compounds of formula (IV) may be linked to such cytotoxic compounds via free $-OH$ or $-NH_2$ groups on such compounds, using methods and reactions described below, or modified using routine experimentation.

Such protected cytotoxic compounds may be useful in GDEPT methods, or *in vitro* assays used to study such methods.

Brief Description of the Drawings

Figure 1 provides a reaction scheme used to make the compounds described in Example 9.

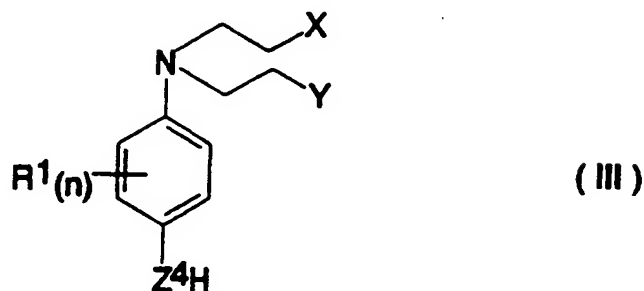
Figure 2 provides a reaction scheme used to make the compounds described in Example 15.

Figure 3 provides a reaction scheme used to make the compounds of the invention described in Examples 1, 2, 3 and 4.

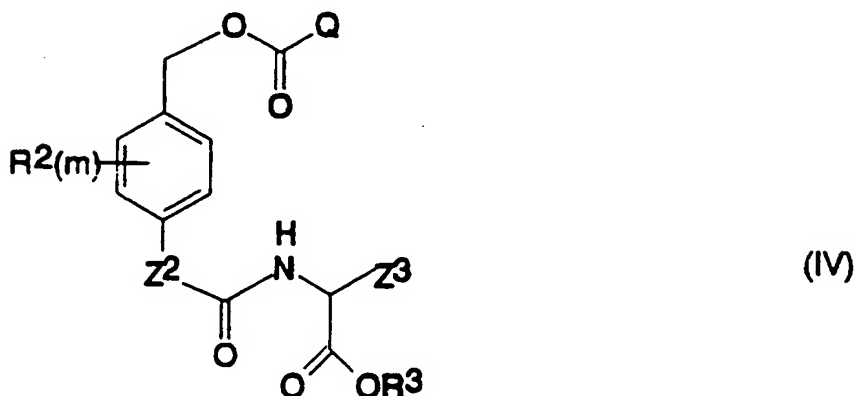
Compounds of the invention may be made using reactions and methods known per se in the art of chemistry. The following methods are of particular use, and are further illustrated by reference to the Examples where indicated. Such reference does not limit the general reaction schemes to those compounds alone. The schemes may be used for other compounds of the invention:

A: Compounds of formula (I) where Z^1 is $-O-$:

(i) Compounds of formula (I) in which $Z^1 = -O-$ and $Z^2 = -NH-$, may be prepared by the reaction of a nitrogen mustard of formula (III)



wherein X, Y, R^1 and n are as defined above and Z^4 is $-O-$ with a linker of formula (IV)



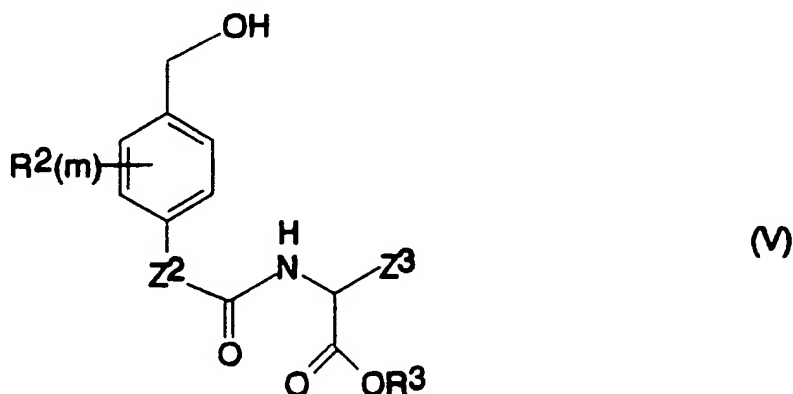
where R^2 , m, R^3 and Z^3 are as defined above, $Z^2 = -NH-$ and Q is a leaving group.

- 5 Preferred leaving groups Q include a succinimidyloxy group, a 4-nitrophenyloxy group, a pentafluorophenyloxy group and a tetrachoroethyloxy $CH(Cl)CCl_3$ group. The reaction may be carried out in aprotic solvents in the presence of a base, for example THF and triethylamine or acetonitrile and basic resins (Example 1).

(ii) Compounds of the formula (III) may be made starting from 4-nitrophenol optionally substituted with the group(s) $R^1_{(n)}$ (as defined above). The phenolic group is

protected as an adamantanyloxycarbonyl-derivative (by reacting the starting materials with adamantanyl-fluoroformate and triethylamine in THF at rt). The protected 4-nitrophenyl carbonate is reduced to the corresponding amine by hydrogen transfer in ethanol using ammonium formate and Pd/C 10% as catalyst at rt. The amine is then hydroxyethylated with ethylene oxide in AcOH at 20°C and then reacted to the desired nitrogen mustard. Reference may be made to EP-A-433 360 or EP-A-490970 for suitable conditions. The compounds may be purified by column chromatography. Deprotection to remove the adamantyl group may be carried out in trifluoroacetic acid.

- (iii) Alternatively, the nitrogen mustard of formula (III) may be activated as a chloroformate by treatment with phosgene or triphosgene in an aprotic solvent and triethylamine followed by coupling with a compound of formula (V):



where R^2 , m , Z^2 , R^3 and Z^3 are as defined above. This may be carried out in THF or other aprotic solvents in the presence of a base (for example triethylamine or pyridine).

- (iv) A further alternative route of synthesis of compounds of the formula (I) in which Z^1 is -O- involves direct coupling of 4-nitrophenol optionally substituted with the group(s) $R^1(m)$ (as defined above) with the compound of the formula (IV) or by reaction of the said optionally substituted 4-nitrophenol compound chloroformate with the compound of formula (VI), in which Z^2 is -NH- (see below) followed in each case by the reaction described above to convert the nitro group, via an amine, to a mustard group.

- (v). A way of obtaining compound (I) where $Z^1 = -O-$ and $Z^2 = -O-$ is from nitrogen mustard (III) where $Z^4 = -O-$, by transforming it to the trimethylsilyl derivative. The silylated phenol is then coupled with a compound of formula (V) where $Z^2 = -O-$. Compound (V) is first activated as the fluoroformate with KF, phosgene and crown ether at -78°C , in an aprotic solvent (see Example 2), prior to reaction with silylated phenol.

B: Compounds of formula (I) where Z^1 is $-NH-$:

- (i) Compounds of the formula (I) in which Z^1 is $-NH-$ may be made by reaction of a compound of formula (III) in which Z^4 is $-NH-$ with a linker of the formula (IV) in aprotic solvents and in the presence of a base. Compounds of the formula (III) in which Z^4 is $-NH-$ may be made from a 1-halo-4-nitrophenyl compound, optionally substituted with the group(s) $R^1_{(m)}$ (as defined above). This is converted to the corresponding 1-bis-hydroxyethylamino-4-nitrophenyl compound by reaction with diethanolamine with heat and the resulting product purified by column chromatography. The corresponding 4-nitro nitrogen mustard may be made by for example mesylation using mesyl chloride in pyridine and subsequent reaction to other halo mustards, e.g. bromo or iodo mustards if required. The 4-nitro group may be reduced by hydrogen transfer in ethanol using ammonium formate and a Pd/C 10% catalyst at 20°C .

- (ii) Alternatively the 1-bis-hydroxyethylamino-4-nitrophenyl compound mentioned above can be reduced using ammonium formate and Pd/C 10% as catalyst in ethanol at 20°C to provide the corresponding phenylene-diamino derivative. This derivative can be converted into the corresponding 4-amino nitrogen mustard as described in the above paragraph, e.g. initially by reaction with mesyl chloride.

- (iii) In an alternative route, the nitrogen mustard (III) $Z^4 = -NH-$ may be activated as an isocyanate by treatment with phosgene or triphosgene in an aprotic solvent followed by coupling with a compound of formula V where R^2 , m , Z^2 , R^3 and Z^3 are as defined above. The coupling may be carried out in aprotic solvents and in the presence of a catalyst, for example dibutyltin dilaurate (see Examples 3 and 4).

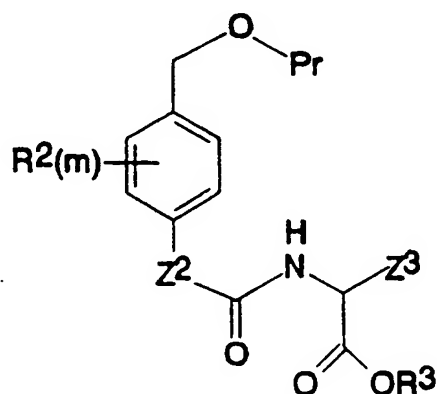
C: Compounds of formula (II):

(i) Compounds of the formula (II) may be obtained by coupling nitrogen mustard phenol compounds described in section A(ii) above with 4-nitrobenzyl chloroformate optionally substituted with the group(s) $R^2_{(m)}$ (as defined above) in the presence or absence of triethylamine at 20°C. This is used to provide compounds of formula (II) in which Z^1 is -O-

(ii) Alternatively aniline nitrogen mustards as described in section B(ii) above may be used with the chloroformate as described in section C(i) above. This is used to provide compounds of the formula (II) in which Z^1 is -NH-.

D: Compounds of the formula (IV) in which Z^2 is -NH-:

(i) Compounds of the formula (IV) in which Z^2 is -NH- may be made from a 4-nitro benzylic alcohol optionally substituted with the group(s) $R^2_{(m)}$ (as defined above). The hydroxyl function is protected as a pyanyl- or *t*-butyl-dimethylsilyl (TBDMSi)-ether by treatment at 20°C with 3,4-dihydropyran and pyridinium-*p*-toluenesulfonate (PPTS) in an aprotic solvent or with TBDMSi chloride and imidazole in dimethylformamide (DMAT), respectively (Example 5). The intermediate thus obtained is reduced to the corresponding amine by hydrogen transfer in ethanol using ammonium formate and Pd/C 10% as catalyst at 20°C. This amine is converted to a glutamyl ester intermediate of formula (VI) (Example 6):



(VI)

where R^2 , m , R^3 and Z^3 are as defined above, Z^2 is $-NH-$ and Pr is the pyanyi- or *t*-butyl-dimethylsilyl (TBDMSi)-ether protecting group. This may be done by treating the amine with triphosgene and triethylamine in toluene at 60°C to provide the corresponding isocyanate, which is coupled with a glutamate derivative of formula $R^3O-C(O)-CH(NH_2)-Z^3$

5 where R^3 and Z are as defined above. Alternatively the corresponding glutamyl-isocyanate obtained from the corresponding glutamate by treatment with triphosgene and triethylamine in toluene at -78°C may be reacted with the amine in a one pot procedure (Example 7). Glutamate derivatives of formula $R^3O-C(O)-CH(NH_2)-Z^3$ may be obtained by reference to WO94/02450. Where necessary or desired, the group Z^3 may be converted to another group
10 Z^3 using routine chemistry known *per se* following production of the compound of formula (VI), or after reaction of that to the compound of formula (I).

(ii) The compound of formula (VI) is deprotected to remove the TBDMSi or pyranil groups by treatment with mild acidic media (AcOH, THF and H_2O or PPTS, EtOH, 55°C) (Example 8). This yields a compound of formula (VI) in which Pr is hydrogen. Compounds
15 of the formula (IV) in which Q is a leaving group may be prepared using standard reactions known in the art (Example 8).

(iii) Where Q is a succinimidyl group the compound of formula (VI) where Pr is hydrogen may be treated with disuccinimidyl-carbonate and triethylamine in acetonitrile. Where a 4-nitrophenyl carbonate group is desired treatment with 4-nitrophenyl chloroformate
20 and triethylamine in THF may be used (Example 9). A pentafluorophenyl carbonate may be added by in situ phosgenation of pentafluorophenol followed by coupling to the linker of formula (VI) in which Pr is hydrogen.

(iv) The compound of formula (VI) in which Pr is hydrogen may be obtained directly by coupling of the 4-aminobenzyl alcohol with glutamyl-isocyanate in aprotic solvents
25 (Example 10).

E: Compounds of the formula (IV) in which Z^2 is $-O-$:

(i) The starting materials for the linkers possessing a carbamic bond are unsubstituted or substituted (with the group(s) $R^2_{(m)}$ (as defined above)) 4-hydroxy-benzyl

alcohols. These type of linkers may require an extra electron withdrawing group on the aromatic nucleus in order to undergo 1,4-elimination. The 4-hydroxy group is specifically protected as an acetate by treating the starting material with acetyl-*v*-triazolo-[4,5-*b*]pyridine, and 1N NaOH in THF at 20°C. The alcohol function of the acetate is further protected as pyran-yl- or TBDMSi-ether by the procedures described in section D above. The acetate function is then deprotected to restore the 4-hydroxy group in NaHCO₃ aq. MeOH at 20°C. The resulting phenol compounds are reacted in a one pot procedure with a protected glutamyl-isocyanate as described in section D(i) above. This yields a compound of the formula of (VI) as shown above in which Z² is -O- and Pr is the pyran-yl- or *t*-butyl-dimethylsilyl (TBDMSi)-ether protecting group.

(ii) Deprotection of this compound yields a compound of the formula (VI) in which Pr is hydrogen. This may be converted to compounds of the formula (IV) by methods analogous to those described in section D (iii) above.

(iii) An alternative procedure starts from 4-hydroxy-benzaldehyde which is protected with 1,3-propane-dithiol in the presence of BF₃·Et₂O, at room temperature, to give the 4{2'-(1',3'-S,S-dithianyl)]phenol. The coupling of the protected phenol with glutamyl isocyanate in toluene in the presence of Et₃N provides the di-*tert*-butyl.O-{4-[2'(1',3'-S,S-dithianyl)]phenyl}oxycarbonyl-L-glutamate. The deprotection of this intermediate to the corresponding aldehyde, is carried out with Hg(ClO₄)₂ in THF at room temperature. The reduction of the aldehyde with a reducing agent like sodium cyano-borohydride yields the desired compound of formula (VI) in which Pr is hydrogen and Z² is -O- (Example 11).

(iv) Another procedure to obtain the deprotected aldehyde is by direct coupling of 4-hydroxy-benzaldehyde with the glutamyl-isocyanate (Example 12).

(v) An alternative route to the compound of formula (VI) in which Z² is -O- and Pr is hydrogen is by direct coupling of the 4-hydroxy-benzylic alcohol, as potassium phenolate (obtained from the starting material and potassium *tert*-butoxyde in the presence of crown ether) with the corresponding glutamyl-isocyanate in aprotic solvents (Example 13).

(vi) The compound of formula (IV) in which Z² is -O- and Q is a leaving group may be prepared using standard reactions known in the art. For example where Q is a 4-nitrophenyl carbonate group the compound of formula (VI) where Pr is hydrogen may be treated with 4-nitrophenyl chloroformate and triethylamine in THF (Example 14).

5 F: Alternative synthesis of compounds of formula (IV):

Compounds of the formula (IV) in which Q is hydrogen, fluoro, chloro, bromo or -O-(N-succinimide) may also be obtained by reference to WO95/03830 published on 9 February 1995 or WO95/02420 published 26 January 1995, the contents of which are hereby incorporated by reference.

10 The following Examples illustrate the invention. The numbering of compounds given in the examples is according to that shown in schemes 1-3.

Example 1.

Diallyl,[(4'-(N,N-bis(2-chloroethyl)amino)phenyloxycarbonyl-oxv-benzyl-4-carbamoyl-L-glutamate] (22)

15 Activated linker 15b (0.20 g, 0.37 mmol) and 4-N,N-bis(2-chloroethyl)aminophenol 21 (0.14 g, 0.60 mmol) was dissolved in 10 mL acetonitrile. Then 0.13 g of a strongly basic resin-
Amberlyst 27 (OH form) was added and the reaction mixture stirred with the resin for 20 h.
The resin was filtered off, and an additional 0.08 g of fresh resin was added. After 30 min
of stirring, the second portion of resin was filtered off, and the solvent evaporated. The
20 product was purified by column chromatography (eluent: cyclohexane:AcOEt 2:1), to afford
22 (82 mg, 32%). ¹H-NMR δ_H: 1.80-2.15 (2m, 2H, CH₂CH(NH)-), 2.30-2.45 (m, 2H,
CH₂CO₂-allyl), 3.72 (s, 8H, N(CH₂CH₂Cl)₂), 4.30-4.40(m, 1H, CH-G), 4.55 (d, 2H,
J=5.41, CH₂O allyl), 4.61 (d, 2H, J=5.31, CH₂O allyl), 5.13 (s, 2H, PhCH₂), 5.10-5.40
(m, 4H, CH₂= allyl), 5.80-6.00 (m, 2H, CH= allyl), 6.64 (d, 1H, J=8.13 Hz, NH-G), 6.75
25 (d, 2H, J=9.10 H_{from2+6}), 7.04 (d, 2H, H_{from3+5}), 7.31 (d, 2H, J=8.55Hz, H_{from2+6}), 7.41
(d, 2H, H_{from3+5}), 8.69 (s, 1H, PhNH). MS (FAB). m/z: 636 (M+1, 60), 658 (M+23, 7).
anal (C₃₀H₃₅N₃O₈Cl₂): calcd C 56.61, H 5.54, N 6.60; found C 56.49, H 5.69, N 6.27.

4'-[N,N-bis(2-chloroethyl)aminolphenyloxycarbonyl-oxy-benzyl-4-carbamoyl-L-glutamic acid (23)

To a solution of **22** (100 mg, 0.16 mmol), PPh_3 (92 mg, 0.35 mmol) and $\text{Pd}(\text{PPh}_3)_4$ (16 mg, 0.014 mmol) in CH_2Cl_2 (8 mL), acetic acid (1 mL) were added and the mixture stirred for 5 h under argon. The solvent was evaporated, and the residue kept under pump vacuum for 2 h, then dissolved in acetone:AcOEt 1:1. The acetone was evaporated affording **23**. PPh_3 as a precipitate in AcOEt. The solid was filtered, washed with AcOEt and then dissolved in 50 mL CHCl_3 and washed with aqueous citric acid 10% (2x50 mL). The organic layer was collected, H_2O was added and the CHCl_3 evaporated yielding **23** (28 mg, 32%) as a precipitate which was filtered from the aqueous solution. $^1\text{H-NMR}$ δ_{H} : 1.80-2.00 (m, 2H, $\text{CH}_2\text{CH}(\text{NH})-$), 2.15-2.25 (t, 2H, $\text{CH}_2\text{CO}_2\text{H}$), 3.72 (s, 8H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 4.10-4.25 (m, PhCH_2), 6.49 (d, 1H, $J=7.34$ Hz, NH-G), 6.75 (d, 2H, $J=9.10$ Hz, $\text{H}_{\text{arom}2'+6'}$), 7.05 (d, 2H, $\text{H}_{\text{arom}3'+5'}$), 7.29 (d, 2H, $J=8.53$ Hz, $\text{H}_{\text{arom}2+6}$), 7.41 (d, 2H, $\text{H}_{\text{arom}3+5}$), 8.75 (s, 1H, PhNH). MS (FAB). m/z : 556 ($\text{M}+1$, 55), 578 ($\text{M}+23$, 100). Accurate mass ($\text{C}_{24}\text{H}_{27}\text{N}_3\text{O}_8\text{Cl}_2\text{Na}$): calcd. 578.1073; found 578.1070; .

Example 2.

4'-[N,N-bis(2-chloroethyl)aminolphenol-trimethylsilyl ether (25)

Phenol nitrogen mustard **21** (60 mg, 0.26 mmol) and Me_3SiCN (40 μL , 0.3 mmol) were stirred at room temperature without solvent for 30 min; then 10 mL boiling hexane was added to the reaction mixture and the remaining solid filtered off. After evaporation of the hexane, **25** (70 mg, 88%) resulted as an oil. $^1\text{H-NMR}$ δ_{H} : 0.20 (s, 9H, $\text{Si}(\text{CH}_3)_3$), 3.55-3.70 (m, 8H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 6.65 (d, 2H, $J=9.30$ Hz, $\text{H}_{\text{arom}2+6}$), 6.73 (d, 2H, $\text{H}_{\text{arom}3+5}$). MS (FAB), m/z : 304 ($\text{M}+$, 100). Accurate mass. ($\text{C}_{13}\text{H}_{21}\text{Cl}_2\text{NOSi}$) calcd. 305.0769; found 305.0765.

diallyl,[(4'-(N,N-bis(2-chloroethyl)amino)phenyloxy-carbonyl-oxy-benzyl-4-oxycarbonyl-L-glutamate] (26)

To a stirred solution of **14b** (100 mg, 0.26 mmol) in CH_2Cl_2 (5 mL) at -78°C , was added phosgene (20% solution in toluene, 150 μL , 0.3 mmol), KF (200 mg, 3.45 mmol) and 18-crown-6 ether (25 μL , 0.12 mmol). After 1 h at -78°C , fluoroformate **24** was formed as indicated by IR spectra ($\nu_{\text{OCOF}}=1827\text{ cm}^{-1}$). Silylated phenol **25** (70 mg, 0.23 mmol) in CH_2Cl_2 (3 mL) was added and the solution stirred 1 h more at -78°C , then filtered and the filtrate evaporated. After purification by preparative HPLC (eluent: cyclohexane:AcOEt 4:1).

26 (60 mg, 41%) resulted. $^1\text{H-NMR}$ δ_{H} : 1.90-2.20 (2m, 2H, $\text{CH}_2\text{CH}(\text{NH})-$), 2.45-2.60 (m, 2H, CH_2CO_2 -allyl), 3.72 (s, 8H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 4.15-4.25 (m, 1H, CH-G), 4.57 (d, 2H, $J=5.40$, CH_2O allyl), 4.63 (d, 2H, $J=5.28$ Hz, CH_2O allyl), 5.23 (s, 2H, PhCH_2), 5.15-5.35 (m, 4H, $\text{CH}_2 =$ allyl), 5.85-6.00 (m, 2H, $\text{CH} =$ allyl), 6.75 (d, 2H, $J=9.11$ Hz, $\text{H}_{\text{arom2}'+6'}$), 7.06 (d, 2H, $\text{H}_{\text{arom3}'+5'}$), 7.14 (d, 2H, $J=8.51$ Hz, $\text{H}_{\text{arom3}'+5'}$), 7.41 (d, 2H, $\text{H}_{\text{arom2}'+6'}$), 8.31 (d, 1H, $J=7.77$, NH-G); MS (FAB) m/z : 637 (M, 100)

4'-(N,N-bis(2-chloroethyl)amino)phenyl-oxy-carbonyl-oxy-benzyl-4-oxycarbonyl-L-glutamic acid (27)

To a solution of 26 (40 mg, 0.063 mmol), PPh_3 (63 mg, 0.24 mmol) and $\text{Pd}(\text{PPh}_3)_4$ (12 mg, 0.01 mmol) in CH_2Cl_2 (3 mL), acetic acid (80 μL) was added and the mixture stirred for 2 h under argon. The solvent was evaporated and the residue dissolved in AcOEt (25 mL), washed with aqueous citric acid 5% (25 mL) and with H_2O (25 mL). The organic layer was dried over MgSO_4 and the solvent evaporated. After purification by preparative HPLC (eluent CH_2Cl_2 : EtOH : AcOH 90:5:5) 27 (8 mg, 25%) resulted. $^1\text{H-NMR}$ (CD_3COOD), δ_{H} : 2.05-2.20 ($\text{CH}_2\text{CH}(\text{NH})-$), 2.61 (t, 2H, $\text{CH}_2\text{CO}_2\text{H}$), 3.60-3.75 (m, 8H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 4.45-4.55 (m, 1H, CH-G), 5.23 (s, 2H PhCH_2), 6.73 (d, 2H, $J=9.20$ Hz, $\text{H}_{\text{arom2}'+6'}$), 7.06 (d, 2H, $\text{H}_{\text{arom3}'+5'}$), 7.19 (d, 2H, $J=8.59$ Hz, $\text{H}_{\text{arom3}'+5'}$), 7.45 (d, 2H, $\text{H}_{\text{arom2}'+6'}$); MS, (ESI) m/z : 557 (M+1, 3).

Example 3.

di-*t*-butyl,[(4'-(N,N-bis(2-chloroethyl)amino)phenylcarbamoyl-oxy-benzyl-4-oxycarbonyl-L-glutamate)] (17)

To a solution of 14a (60 mg 0.15 mmol) in toluene (5 mL) was added isocyanate 16 (0.60 mmol) in toluene (5 mL) and dibutyltin dilaurate (10 μL). The reaction mixture was stirred for 12 h at room temperature. The solid which formed was filtered off and the filtrate evaporated. Purification was achieved by column chromatography (eluent: cyclohexane: AcOEt 3:1), when product 17 (90 mg, 90%) was obtained as an oil. $^1\text{H-NMR}$ δ_{H} : 1.41 (s, 9H, *t*-Bu), 1.42 (s, 9H, *t*-Bu), 1.65-2.05 (2m, 2H, $\text{CH}_2\text{CH}(\text{NH})-$), 2.30-2.40 (m, 2H, CH_2CO_2 -*t*-Bu), 3.68 (s, 8H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 3.95-4.15 (m, 1H, CH-G), 5.10 (s, 2H, PhCH_2), 6.70 (d, 2H, $J=8.75$ Hz, $\text{H}_{\text{arom3}'+5'}$), 7.10 (d, 2H, $J=8.31$ Hz, $\text{H}_{\text{arom3}'+5'}$), 7.28 (d, 2H, $\text{H}_{\text{arom2}'+6'}$), 7.42 (d, 2H, $\text{H}_{\text{arom2}'+6'}$), 8.92 (d, 1H, $J=7.80$ Hz, NH-G), 9.36 (s, 1H, $\text{Ph}'\text{-NH}$). MS, m/z (FAB): 667 (M+1, 5), 689 (M+23, 100)

4'-[N,N-bis(2-chloroethyl)amino]phenylcarbamoyl-oxy-benzyl-4-oxycarbonyl-L-glutamic acid (18)

Di-*t*-butyl ester 17 (90 mg, 0.135 mmol) was dissolved in formic acid (10 mL) and stirred under argon at 12 °C for 24 h. The reaction mixture was lyophilised, the freeze-dried material triturated with CH₂Cl₂, the solvent removed and the solid kept in a dessicator 24 h under vacuum to afford 18 (65 mg, 87%). ¹H-NMR δ_H: 1.75-2.15 (2m, 2H, CH₂CH(NH)-), 2.30-2.50 (m, 2H, CH₂CO₂H), 3.68 (s, 8H, N(CH₂CH₂Cl)₂), 3.95-4.10 (m, 1H, CH-G), 5.10 (s, 2H, PhCH₂), 6.70 (d, 2H, J=8.66Hz, H_{arom3'+5'}), 7.11 (d, 2H, J=8.29, H_{arom3'+5'}), 7.28 (d, 2H, H_{arom2'+6'}), 7.42 (d, 2H, H_{arom2'+6'}), 8.07 (d, 1H, J=7.80 Hz, NH-G), 9.36 (s, 1H, Ph'NH). MS, m/z (FAB): 555 (M, 90), 578 (M+23, 100). Accurate mass: calcd: (C₂₄H₂₇N₃O₈Cl₂Na), 578.1073, found: 578.1070.

Example 4.

diallyl,[(4'-[N,N-bis(2-chloroethyl)amino]phenylcarbamoyl-oxy-benzyl-4-carbam yl-L-glutamate] (19)

A similar procedure as for 17 was used, starting from isocyanate 16 (0.54 mmol) in toluene (5 mL). 8b (0.10 g, 0.27 mmol) dissolved in CH₂Cl₂ (5 mL), and dibutyltin dilaurate (10 μL), to yield 19 as a solid. After further recrystallisation from hexane:AcOEt 9:2 19 (0.11 mg, 64%) resulted. ¹H-NMR δ_H: 1.75-2.15 (2m, 2H, CH₂CH(NH)-), 2.35-2.50 (m, 2H, CH₂CO₂-Allyl), 3.68 (s, 8H, N(CH₂CH₂Cl)₂), 4.25-4.40(m, 1H, CH-G), 4.55 (d, 2H, J=5.39 Hz, CH₂O allyl), 4.61 (d, 2H, J=5.37 Hz, CH₂O allyl), 5.01 (s, 2H, PhCH₂), 5.15-5.35 (m, 4H, CH₂= allyl), 5.80-6.05 (m, 2H, CH= allyl), 6.61 (d, 1H, J=7.97 Hz, NH-G), 6.69 (d, 2H, J=8.99Hz, H_{arom3'+5'}), 7.28 (d, 4H, J=7.79 Hz, , H_{arom2'+6'}+H_{arom2'+6'}), 7.39 (d, 2H, H_{arom3'+5'}), 8.64 (s, 1H, PhNH,), 9.30 (s, 1H, Ph'NH). MS, m/z (FAB): 635 (M+1, 5), 657 (M+23, 5).

4'-[N,N-bis(2-chloroethyl)amino]phenylcarbamoyl-oxy-benzyl-4-carbamoyl-L-glutamic acid (20)

Diallyl ester 19 (40 mg, 0.06 mmol) dissolved in CH₂Cl₂ (2 mL) was stirred with Pd(PPh₃)₄ (4 mg) and pyrrolidine (15 μL, 0.15 mmol) The reaction mixture was kept under argon for 45 min then diluted with 2 mL toluene. The pyrrolidine salt of the acid 20 precipitated and was recovered by filtration. The free acid was obtained by extracting the precipitate with CH₂Cl₂/aqueous citric acid 10%. The organic layer was washed with H₂O and evaporated to

give the free acid (20 mg, 60%) after filtration of the remaining water. $^1\text{H-NMR}$ δ_{H} : 1.70-2.05 (2m, 2H, $\text{CH}_2\text{CH}(\text{NH})-$), 2.20-2.35 (m, 2H, CH_2CO_2 -allyl), 3.68 (s, 8H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 4.15-4.30 (m, 1H, CH-G), 5.01 (s, 2H, PhCH_2), 6.45 (d, 1H, $J=7.98\text{Hz}$, NH-G), 6.69 (d, 2H, $J=9.05\text{Hz}$, $\text{H}_{\text{arom}3'+5'}$), 7.27 (d, 4H, $J=8.60\text{Hz}$, $\text{H}_{\text{arom}2'+6'} + \text{H}_{\text{arom}2'+6'}$), 7.39 (d, 2H, $J=8.52$, $\text{H}_{\text{arom}3'+5'}$), 8.65 (s, 1H, PhNH), 9.30 (s, 1H, $\text{Ph}'\text{NH}$). MS (FAB). m/z : 554 (M, 50), 577 (M+23, 100). Accurate mass: calcd: ($\text{C}_{24}\text{H}_{23}\text{N}_4\text{O}_7\text{Cl}_2\text{Na}$) 577.1233, found: 577.1230.

Example 5.

(4-nitro-benzyl) *t*-butyl-di-phenyl-silyl ether (2a).

To a stirred solution of 4-nitrobenzyl alcohol (1.00 g, 6.50 mmol) and imidazole (0.97 g, 14.10 mmol) in DMF (10.0 mL), *t*-butyl-diphenyl-chlorosilane (1.98 g, 1.87 mL, 7.20 mmol) was added over 10 min under nitrogen at room temperature. The reaction mixture was stirred for an additional 5 h, diluted with 75 mL Et_2O , washed with H_2O (5 times, 15 mL), dried (MgSO_4) and evaporated under vacuum. An oil which crystallised on standing, resulted: yield: 2.54 g. After recrystallisation from EtOH 70%, 2.36 g (93%) of a white solid is obtained. mp 80-81 $^\circ\text{C}$. $\nu_{\text{max}}/\text{cm}^{-1}$ (film): 2931, 2857 (CH_2 , asym., sym.), 1521, 1345 (NO_2); $^1\text{H-NMR}$, δ_{H} : 1.06 (9H, s, *t*-Bu), 4.92 (2H, s, CH_2), 7.42-7.46 (5H, m, Ph), 7.63-7.65 (7H, m, Ph + $\text{H}_{\text{arom}2'+6'}$), 8.23, (2H, d, $J = 8.23\text{ Hz}$, $\text{H}_{\text{arom}3'+5'}$); MS, (EI), (391.54); m/z : 334 (M - *t*-Bu, 100), 288 (M - *t*-Bu - NO_2 , 10), 256 (M - *t*-Bu - Ph, 20), 199 (Ph_2SiOH^+ , 100); $\text{C}_{13}\text{H}_{15}\text{NO}_3\text{Si}$.

20 4-nitro-benzyl -2'-tetrahydropyranyl ether(2b).

To a stirred solution of 4-nitrobenzyl alcohol (1.53 g, 10.0 mmol) in CH_2Cl_2 (50 mL), 3,4-dihydropyran (1.36 mL, 1.26 g, 15.0 mmol) and pyridinium-*p*-toluene sulphonate (0.25 g) were added. After 1.5 h the reaction mixture was evaporated to 4-5 mL, AcOEt (25 mL) was added and the solution washed with water (3x50mL), then brine, dried (MgSO_4) and evaporated under vacuum. The product, 2b, (2.46 g, 99%) resulted as an oil. $^1\text{H-NMR}$, δ_{H} : 1.51-1.63 (m, 4H, $2\text{H}_4 + 2\text{H}_5$), 1.66-1.74 (m, 1H, 2H_3), 3.50 (s, 1H, H_6), 3.78 (m, 1H, H_6), 4.62 (d, 1H, $J=3.8\text{ Hz}$, CH_2 -Ph), 4.72 (m, 1H, H_2), 4.81 (d, 1H, CH_2 -Ph), 7.62 (d, 2H, $J=8.5\text{ Hz}$, H_{arom}), 8.22 (d, 2H, H_{arom}); MS, (FAB), (237.52); m/z : 260 ($\text{M}^+ + 23$, 15), 236 (M - 1, 45), 136 ($4\text{-NO}_2\text{C}_6\text{H}_4\text{CH}_2^+$, 100).

Example 6.

(4-amino-benzyl) *t*-butyl-di-phenyl-silyl ether (3a)

To a stirred solution of **2a** (5.00 g, 12.80 mmol) in ethanol (100 mL) was added Pd/C 10% (1.50 g) and ammonium formate (4.60 g) in one portion at 20 °C. After 1.5 h the catalyst was removed by filtration, the filtrate concentrated under vacuum and the residue partitioned between EtOAc:H₂O. The organic layer was dried (MgSO₄) and concentrated to give **3a** as an oil; yield: 4.24 g (91 %); $\nu_{\text{max}}/\text{cm}^{-1}$ (film): 3433, 3378 (NH₂), 2931, 2857 (CH₂, asym., sym.); ¹H-NMR, δ_{H} : 1.00 (s, 9H, *t*-Bu), 4.57 (s, 2H, CH₂), 4.98 (s broad, 2H, NH₂), 6.52 (d, 2H, J = 8.25 Hz, H_{arom3+5}), 6.96 (d, 2H, H_{arom2+6}), 7.42-7.46 (m, 5H, Ph), 7.62-7.65 (m, 5H, Ph); MS, (EI), (361.56); m/z: 361 (M⁺, 8), 304 (M - *t*-Bu, 100), 199 (Ph₂SiOH⁺, 100); anal (C₂₃H₂₇NOSi), C, H, N.

(4-amino-benzyl)-O-2'-tetrahydropyranyl ether (3b)

A similar method as for **3a** was employed using **2b** (8.53 g, 35.9 mmol) in ethanol (150 mL), Pd/C 10% (3.50 g) and ammonium formate (12.0 g, 180.0 mmol). An oil (6.40g, 36%) resulted after work up, which was purified by column chromatography (cyclohexane:AcOEt 1:1): The desired compound eluted first as an oil (2.59 g, 38%). $\nu_{\text{max}}/\text{cm}^{-1}$ (film): 3447, 3360 (NH₂, sym, asym), 2943, 2870 (CH₂, asym., sym.); ¹H-NMR, δ_{H} : 1.42-1.53 (m, 4H, 2H₁+2H₂), 1.55-1.72 (m, 1H, 2H₃), 3.45 (s, 1H, H₄), 3.79 (m, 1H, H₄), 4.22 (d, 1H, J=11.1 Hz, CH₂-Ph), 4.37 (d, 1H, CH₂-Ph), 4.60 (d, 1H, H₂), 5.00 (s, 2H, NH₂), 6.52 (d, 2H, J=8.23 Hz, H_{arom}), 6.97 (d, 2H, H_{arom}); MS, (EI), (207, 27); m/z: 207 (M⁺, 100), 106 (H₂NC₂H₃CH₂⁺, 100); anal (C₁₇H₁₇NO₂), C, H, N.

Example 7.

di-*t*-butyl, (*t*-butyl-di-phenyl-silyl-O-benzyl)-4-carbamoyl-L-glutamate (7a)

Method A: A suspension was formed by adding 0.31 g (0.43 mL, 3.1 mmol) triethylamine to a solution of di-*t*-butyl-glutamate hydrochloride (0.46 g, 1.55 mmol) in 7 mL THF. The isocyanate, **4a**, (0.60 g, 1.55 mmol) dissolved in 3 mL THF (dry) was added to this at room temperature. After 2 h the precipitate was filtered and the solvent removed under vacuum, giving 0.87 g (87%) of an oil. The product was purified by column chromatography (AcOEt: cyclohexane 2:1) yielding 0.53 g (53%) of pure **7a**, as a white solid, mp=89-90 °C. $\nu_{\text{max}}/\text{cm}^{-1}$ (film): 3359 (NH), 2932, 2857 (CH₂, asym., sym.), 1729 (C=O, ester), 1670 (C=O, urea), 1154 (C-O, str.); ¹H-NMR, δ_{H} : 1.03 (s, 9H, *t*-Bu), 1.10 (s, 9H, *t*-Bu-glu), 1.43 (s, 9H, *t*-Bu-glu), 1.68-2.00 (2m, 2H, CH(NH)CH₂), 2.18-2.32 (m, 2H, CH₂CO₂-*t*-Bu), 4.08-4.12 (m,

¹H. CH(NH)CH₂). 4.68 (s, 2H, PhCH₂), 6.38 (d, 1H, J = 8.12 Hz, NH-glu), 7.19 (d, 2H, J = 8.41 Hz, H_{arom3+5}), 7.32-7.47 (m, 7H, Ph+H_{arom2+6}), 7.62-7.70 (m, 5H, Ph), 8.54 (s, 1H, NH-Ph); MS, (EI), (646.90); m/z: 590 (M - *t*-Bu + 1, 2), 534 (M - 2*t*-Bu + 2, 5), 478 (M - 3*t*-Bu + 3, 100), 199 (Ph₂SiOH⁺, 100); anal (C₃₇H₅₀N₂O₆Si), C, H, N.

Method B: One pot synthesis of compound 7a : To a solution of di-*t*-butyl-glutamate hydrochloride (4.14 g, 14.0 mmol) and triphosgene (1.39 g, 4.67 mmol) in toluene, cooled at -78 °C. 3.90 mL (2.83 g, 28 mmol) triethylamine in 10 mL toluene were added dropwise over 30 min. The reaction was allowed to reach room temperature and was completed by 1 h. To this mixture, a solution containing (4-amino-benzyl)-*t*-butyl-diphenyl-silyl ether 3a (5.00 g, 13.8 mmol) and triethylamine (1.95 mL, 14.0 mmol) was added over 5-10 min. The reaction was completed by 20 h. The reaction mixture was filtered, washed with H₂O (200 mL), HCl solution 1 % (200 mL), Na₂CO₃ solution 1 % (200 mL), H₂O (twice, 200 mL), dried (MgSO₄) and evaporated under vacuum, yielding a thick oil. Yield: 9.90 g. (TLC, AcOEt : cyclohexane 3:1). The product was used for deprotection without further purification.

diallyl-(2'-tetrahydropyranyl-O-benzyl)-4-carbamoyl-L-glutamate (7b)

A similar one pot procedure was used, starting from diallyl glutamate-*p*-toluene-sulphonate (1.08 g, 3.1 mmol), triphosgene (0.30 g, 1.03 mmol) and triethylamine (0.86 mL, 6.2 mmol) in toluene (20 mL) at -78 °C to prepare the diallyl-L-glutamyl isocyanate 6b. To the reaction mixture 3b (0.52 g, 2.5 mmol) and triethylamine (0.41 mL) in 5 mL toluene was added. An oil resulted (1.17 g) which was separated by preparative HPLC using cyclohexane:AcOEt 1.5:1 as eluent. The desired compound separated as an oil (0.75 g, 65%).
 $\nu_{\text{max}}/\text{cm}^{-1}$ (film): 3362 (NH₂), 2944, 2871 (CH₂, asym., sym.); ¹H-NMR, δ_{H} : 1.47-1.50 (m, 4H, 2H₂ + 2H₃), 1.60-1.68 (m, 3H, 2H₃ + CH₂CH(NH)-), 1.88-2.05 (m, 1H, -CH₂CH(NH)-), 2.37-2.45 (m, 2H, CH₂CO₂A), 3.45 (s, 1H, H₆), 3.80 (m, 1H, H₆), 4.20-4.31 (m, 1H, -CH(NH)-), 4.35 (d, 1H, J=11.55 Hz, CH₂-Ph), 4.53-4.63 (m, 5H, CH₂-Ph+CH₂O allyl), 5.18-5.33 (m, 4H, CH₂= allyl), 5.80-6.00 (m, 2H, CH= allyl), 6.60 (d, 1H, J=8.03 Hz, NH-G), 7.20 (d, 2H, J=8.47 Hz, H_{arom}), 7.35 (d, 2H, H_{arom}), 8.47 (s, 1H, NH-Ph); MS, (EI), (460, 53); anal (C₂₄H₃₂N₂O₇), C, H, N.

Example 8.

di-*t*-butyl,(4-hydroxybenzyl)carbamoyl-L-glutamate (8a)

Tetra-butylammonium fluoride solution in THF (1M) 20.0 mL (2.5 eq) was added to compound 7a (5.15 g, 8.0 mmol) in 100 mL THF at room temperature. The reaction was finished in 3 h (TLC AcOEt : cyclohexane 3:1). The reaction mixture was evaporated under vacuum. The oil thus obtained was dissolved in AcOEt (50mL), washed with water (3x100 mL), dried (MgSO₄) and evaporated again. An oil (5.08 g) was obtained. The deprotected compound, 8a, was purified by column chromatography (AcOEt : cyclohexane 3:1) yielding 1.88g (58.5%) of an oil which crystallised on standing. Recrystallisation from 60% aqueous MeOH afforded a white solid. Mp : 103-104 °C; $\nu_{\max}/\text{cm}^{-1}$ (film): 3370 (broad, NH+OH), 2967 (CH₃), 2930, 2857 (CH₂, asym., sym.), 1716 (C=O, ester), 1678 (C=O, amide), 1153 (C-O, str.); ¹H-NMR, δ_{H} : 1.40 (s, 9H, *t*-Bu), 1.42 (s, 9H, *t*-Bu), 1.72-2.00 (2m, 2H, CH(NH)CH₂), 2.20-2.31 (m, 2H, CH₂CO₂-*t*-Bu), 4.10-4.18 (m, 1H, CH(NH)CH₂), 4.39 (d, 2H, J = 5.36 Hz, CH₂), 4.99 (t, 1H, CH₂OH), 6.38 (d, 1H, J = 8.11 Hz, NH-glut), 7.16 (d, 2H, J = 8.35 Hz, H_{arom3+5}), 7.31 (d, 2H, H_{arom2+6}), 8.50 (s, 1H, NH-Ph); MS, (EI), (408.49); m/z: 408 (M⁺, 10), 352 (M - *t*-Bu + 1, 4), 296 (M - 2*t*-Bu + 2, 14); anal (C₂₁H₃₂N₂O₆, C, H, N).

diallyl,(4-hydroxybenzyl)carbamoyl-L-glutamate (8b)

The intermediate 7b (0.61 g, 1.3 mmol) was hydrolysed at 45 °C in 24.5 mL of a mixture AcOH:THF:H₂O 4:2:1 for 3.5 h. (monitored by TLC, eluent, AcOEt:cyclohexane 1:1). The reaction mixture was diluted with 50 mL H₂O, extracted with ether (2x25 mL), then with AcOEt (2x30 mL). The pooled organic layers were washed with water (2x30 mL), dried (MgSO₄) and evaporated to dryness (with addition of toluene, 2x30mL). An oil resulted (0.52 g) which was purified by preparative HPLC (cyclohexane:AcOEt 1:2), yielding compound 8b (0.221 g, 45%). $\nu_{\max}/\text{cm}^{-1}$ (film): 3354 (NH₂, OH broad), 1737 (C=O, ester), 1659 (C=O, amide); ¹H-NMR, δ_{H} : 1.85-1.93 (m, 1H, CH₂CH(NH)-), 2.00-2.05 (m, 1H, -CH₂CH(NH)-), 2.46 (t, 2H, J=5.42 Hz, CH₂CO₂A), 4.26-4.35 (m, 1H, -CH(NH)-), 4.40 (d, 2H, J=5.64 Hz, CH₂-Ph), 4.55 (d, 2H, J=5.32 Hz, CH₂O allyl), 4.61 (d, 2H, CH₂O allyl), 4.98 (t, 1H, OH), 5.17-5.37 (m, 4H, CH₂= allyl), 5.85-5.94 (m, 2H, CH= allyl), 6.56 (d, 1H, J=8.03 Hz, NH-G), 7.17 (d, 2H, J=8.48 Hz, H_{arom}), 7.32 (d, 2H, H_{arom}), 8.52 (s, 1H, NH-Ph); MS, (EI), (376.41); m/z: 399 (M⁺+23, 35), 377 (M⁺+1, 100), 359 (M⁺-43, 34), anal (C₁₆H₂₄N₂O₆, C, H, N).

Example 9.

di-*t*-butyl,[4(4'-nitrophenyloxy-carbonyl)oxybenzyl]-carbamoyl-L-glutamate (9a)

To a stirred solution of 8a (0.20 g, 0.49 mmol) in dry THF (10 mL) were added 0.11 g (0.5 mmol) 4-nitrophenyl chloroformate and triethylamine (0.1 mL, 0.6 mmol) at room temperature. The reaction was complete by 1h. The precipitate formed was filtered and the solution concentrated under vacuum. AcOEt (10 mL) was added, the solution washed with brine (2x10 mL), dried and evaporated again, giving an oil (0.28 g). The product was purified by column chromatography (0.16 g, 57%) and repurified by preparative HPLC (0.14 g). $\nu_{\max}/\text{cm}^{-1}$ (film): 3349 (NH₂), 2979, 2932 (CH₂, asym., sym.), 1767 (C=O, carbonate), 1716 (C=O, ester), 1652 (C=O, amide), 1527, 1349 (NO₂); ¹H-NMR, δ_{H} : 1.39 (9H, s, *t*-Bu), 1.42 (9H, s, *t*-Bu), 1.67-2.05 (2H, 2m, CH₂CH(NH)-), 2.20-2.38 (2H, 2m, CH₂CO₂-*t*-Bu), 4.08-4.21 (1H, m, CH(NH)-), 4.63 (2H, s, CH₂Ph), 6.46 (1H, d, J = 7.99 Hz, NH-glu), 7.33 (2H, d, J = 8.61 Hz, H_{arom3+5}), 7.42 (2H, d, H_{arom2+6}), 7.56 (2H, d, J = 9.17, H_{arom1}), 8.30 (2H, d, H_{arom2+6}), 8.68 (1H, s, NH-Ph); MS. (EI), (573, 60); anal (C₂₈H₃₅N₃O₁₀), C, H, N.

diallyl,[4(4'-nitrophenyloxy-carbonyl)oxybenzyl]-carbamoyl-L-glutamate (9b)

Starting from 8b (0.19 g, 0.50 mmol), 9b was obtained by a similar procedure and purified by preparative HPLC. Compound 9b resulted as a white solid (0.132 g, 48.6%). ¹H-NMR, δ_{H} : 1.86-1.95 (m, 1H, CH₂CH(NH)-), 2.03-2.12 (m, 1H, -CH₂CH(NH)-), 2.46 (t, 2H, J=7.40 Hz, CH₂CO₂A), 4.33-4.48 (m, 1H, -CH(NH)-), 4.55 (d, 2H, J=5.33 Hz, CH₂O allyl), 4.61 (d, 2H, CH₂O allyl), 5.17-5.38 (m, 4H, CH₂= allyl), 5.22 (s, 2H, CH₂Ph), 5.87-5.96 (m, 2H, CH= allyl), 6.65 (d, 1H, J=8.26 Hz, NH-G), 7.34 (d, 2H, J=8.67 Hz, H_{arom}), 7.43 (d, 2H, H_{arom}), 7.56 (d, 2H, J=9.14 Hz, H_{arom}), 8.31 (d, 2H, H_{arom}), 8.71 (s, 1H, NH-Ph); MS, (EI), (541,); m/z: 564 (M⁺+23, 60), 542 (M⁺+1, 6); anal (C₂₆H₂₇N₃O₁₀): C, H, N; accurate mass: calcd. (C₂₆H₂₇N₃O₁₀Na): 564.1594; found 564.1590.

Example 10.

diallyl,(4-hydroxybenzyl)carbamoyl-L-glutamate (8b)

To a solution of diallyl, glutamyl isocyanate 6b, [prepared from diallyl glutamate-*p*-toluene sulphonate (10.0 g, 25.0 mmol), triphosgene (2.46 g, 8.3 mmol) and triethylamine (10.5 mL, 75.0 mmol, 3 eq) in 100 mL THF, at -78 °C] was added dropwise 4-aminobenzyl alcohol, 28. (3.0 g, 24.3 mmol) and triethylamine (3.41 mL, 24.3 mmol) in 20 mL toluene, over 10

min at room temperature. The reaction was complete by 15 min. The reaction mixture was filtered, evaporated to dryness and the residue dissolved in 20 mL AcOEt, washed with water (2x20 mL), dried (MgSO₄) and re-evaporated. An oil resulted (10,25 g). 2.7 g of the obtained product was submitted to purification by preparative HPLC when 1.52 g (63 %) of pure 8b resulted

Example 11.

di-*t*-butyl, {4[2'(1'3'-S,S-dithienyl)phenyl]oxycarbonyl-L-glutamate} (12).

To a solution of di-*t*-butyl-glutamyl isocyanate 6a (prepared from di-*t*-butyl-glutamate hydrochloride (1.50 g, 5.0 mmol), triphosgene (0.50 g, 1.66 mmol) and triethylamine (1.40 mL, 28.0 mmol) in 50 mL toluene, at -78 °C), was added 4[2'(1'3'-S,S-dithienyl}phenol, 11 (1.10g, 5.0 mmol) and triethylamine (0.70 mL). After 12 h, the solid formed was filtered and the solution washed with: NaOH 1% (75 mL), water (2x75 mL), dried (MgSO₄) and evaporated. A white solid (2.30 g, 95.8%) was obtained. Recrystallisation from aqueous EtOH 50% afforded the pure 12 (1.81 g, 72.7%). $\nu_{\max}/\text{cm}^{-1}$ (film): 3347 (NH₂), 2977, 2945 (CH₂, asym., sym.), 1728 (C=O, ester); ¹H-NMR, δ_{H} : 1.41 (s, 9H, *t*-Bu), 1.42 (s, 9H, *t*-Bu), 1.62-1.90 (m, 2H, CH₂-5'), 1.90-2.32 (2m, 2H, CH₂CH(NH)-), 2.30-2.40 (m, 2H, CH₂CO₂-*t*-Bu), 2.86-2.93 (m, 2H, CH₂-4' or 6'), 3.09 (t, 2H, J=12.3 Hz, CH₂-6' or 4'), 5.41 (s, 1H, H-2'), 7.08 (d, 2H, J = 8.50 Hz, H_{arom3-5}), 7.43 (d, 2H, H_{arom2+6}), 8.12 (d, 1H, J=7.81 Hz, NH-Ph); MS, (EI), (497, 67); m/z: 497 (M⁺, 2), 451 (M - *t*-Bu, 2), 359 (M - 2*t*-Bu, 20); Anal.: (C₂₄H₂₅NO₆S₂), C, H, N.

di-*t*-butyl, (4-benzaldehyl)oxycarbonyl-L-glutamate (13a).

To the solution of dithiane 12 (0.50 g, 1.0 mmol) in CHCl₃ (10 mL) and THF (5 mL) was added dropwise a solution of Hg(ClO₄)₂·3H₂O (0.91 g, 2.0 mmol) in 5mL THF. After 5 min the precipitate was filtered and the solution is washed with H₂O (2x25 mL), sodium carbonate (25 mL), H₂O (2x25 mL), dried (MgSO₄) and evaporated. The compound 13a (0.33g, 81%) was obtained as a clear oil. $\nu_{\max}/\text{cm}^{-1}$ (film): 3347 (NH₂), 2979, 2934 (CH₂, asym., sym.), 1731 (C=O, ester), 1712 (C=O, aldehyde); ¹H-NMR, δ_{H} : 1.41 (s, 9H, *t*-Bu), 1.43 (s, 9H, *t*-Bu), 1.80-1.87 (m, 1H, CH₂CH(NH)-), 1.96-2.02 (m, 1H, CH₂CH(NH)-), 2.32-2.39 (m, 2H, CH₂CO₂-*t*-Bu), 3.99-4.04 (m, 1H, CH-G), 7.34 (d, 2H, J = 8.39 Hz, H_{arom3+5}), 7.96 (d, 2H, H_{arom2+6}), 8.29 (d, 1H, J=7.72 Hz, NH-Ph), 9.98 (s, 1H, CHO):

di-*t*-butyl,(4-hydroxybenzyl)oxycarbonyl-L-glutamate (14a)

Compound 13a (0.150 g, 0.36 mmol) dissolved in 5.2 mL of a mixture of H₂O:AcOH:CH₃OH (1:1:1) was reduced with sodium-cyanoborohydride (0.027 g, 0.43 mmol) at room temperature. After 1h, AcOEt (20 mL) was added, the reaction mixture
 5 evaporated, the residue dissolved in AcOEt (20 mL), the solution washed with H₂O (20 mL),
 brine (2x20 mL), dried and evaporated to dryness. Compound 14a (0.127g, 86%) was
 obtained as an oil. The final purification was achieved by preparative HPLC
 (cyclohexane:AcOEt, 2:1). $\nu_{\max}/\text{cm}^{-1}$ (film): 3352 (NH₂, OH, broad), 2979, 2945 (CH₂,
 asym., sym.), 1728 (C=O, ester), 1712 (C=O, aldehyde); ¹H-NMR, δ_{H} : 1.41 (s, 9H, *t*-Bu),
 10 1.42 (s, 9H, *t*-Bu), 1.70-1.82 (m, 1H, CH₂CH(NH)-), 1.94-1.99 (m, 1H, CH₂CH(NH)-),
 2.31-2.38 (m, 2H, CH₂CO₂-*t*-Bu), 3.97-4.02 (m, 1H, CH-G), 4.48 (d, 2H, J = 5.70 Hz,
 PhCH₂OH), 5.15 (t, 1H, OH), 7.03 (d, 2H, J = 8.39 Hz, H_{arom3+5}), 7.31 (d, 2H, H_{arom2+6}),
 8.04 (d, 1H, J=7.88 Hz, NH-Ph), 9.98 (s, 1H, CHO); anal: (C₂₁H₃₁NO₇), C, H, N.

Example 12.

di-*t*-butyl,(4-benzaldehyd)oxycarbonyl-L-glutamate (13a).

To a solution of di-*t*-butyl-glutamyl isocyanate 6a [prepared from di-*t*-butyl-glutamate
 hydrochloride (0.200 g, 0.67 mmol), triphosgene (0.067 g, 0.22 mmol) and triethylamine
 (0.190 mL, 1.35 mmol) as above] in 15 mL CHCl₃, were added 4-hydroxybenzaldehyde
 (0.082g, 0.67 mmol) and triethylamine (0.190 mL, 1.35 mmol) in 10 mL CHCl₃ over 10
 20 min. The reaction was heated under reflux for 2 h, then the solvent was removed under
 vacuum. The compound was purified by preparative HPLC (eluent: cyclohexane:AcOEt 6:1).
 Finally a clear oil was obtained (0.197 g, 72%).

Example 13.

diallyl,(4-hydroxybenzyl)oxycarbonyl-L-glutamate (14b)

A clear solution of diallyl-glutamyl isocyanate 6b [prepared from diallyl-glutamate p-toluene
 sulphate (3.28 g, 8.20 mmol), triphosgene (0.81 g, 2.73 mmol) and triethylamine (3.44
 mL, 24.6 mmol) in 50 mL THF, at -78 °C, according to the procedure described above] was
 obtained by filtering the reaction mixture at room temperature. 4-hydroxybenzyl alcohol 29
 (1.00 g, 8.10 mmol) was dissolved in THF (dry) (10 mL) and 18-crown-6 ether (0.20 mL)
 30 and potassium tert-butoxide (0.16 g, 1.4 mmol) were added. To this mixture, the solution of
 isocyanate 6b was added dropwise at room temperature with vigorous stirring. The reaction

was terminated by addition of AcOH (0.5 mL). The reaction mixture was filtered and the solvent removed under vacuum. The residue was dissolved in AcOEt (20 mL), the solution washed with aqueous NaOH 4% (20 mL), with H₂O (2x20 mL), dried and evaporated to dryness. Compound 14b (1.62g, 53 %) resulted and was purified by preparative HPLC (eluent cyclohexane:AcOEt 1:1). Finally a clear oil was obtained (0.80 g, 26.5%). ¹H-NMR, δ_H : 1.90-2.01 (m, 1H, CH₂CH(NH)-), 2.05-2.09 (m, 1H, -CH₂CH(NH)-), 2.50-2.56 (m, 2H, CH₂CO₂A), 3.96-4.05 (m, 1H, -CH(NH)-), 4.48 (d, 2H, J=5.67 Hz, PhCH₂OH), 4.58 (d, 2H, J=5.26 Hz, CH₂O allyl), 4.63 (d, 2H, CH₂O allyl), 5.16 (t, 1H, OH), 5.19-5.38 (m, 4H, CH₂= allyl), 5.95-5.99 (m, 2H, CH= allyl), 7.04 (d, 2H, J=8.47 Hz, H_{arom3+5}), 7.32 (d, 2H, H_{arom2+6}), 8.24 (d, 1H, J=7.82 Hz, NH-Ph); MS, (FAB), (377,); m/z: 400 (M⁺+23, 82), 378 (M⁻+1, 3); accurate mass (C₁₉H₂₃NO₇Na): calc. 400.1372; found 400.1376

Example 14.

di-*t*-butyl, [4(4'-nitrophenyloxycarbonyl)oxybenzyl]-oxycarbonyl-L-glutamate (15a)

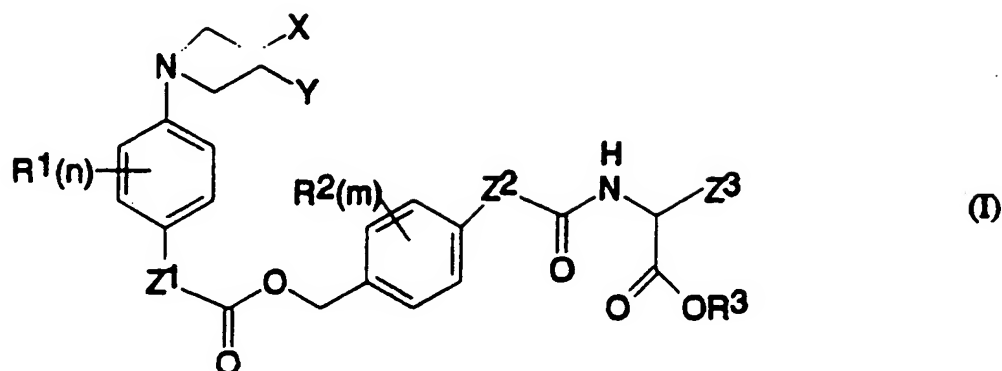
Activation of linker 14a (1.0 g, 2.7 mmol) was achieved by the same procedure as in Example 9. After purification, an oil resulted (0.64 g, 41%). ν_{max}/cm^{-1} (film): 3367 (NH₂), 2980, 2933 (CH₂, asym., sym.), 1767 (C=O, carbonate), 1728 (C=O, ester); ¹H-NMR, δ_H : 1.41 (s, 9H, *t*-Bu), 1.43 (s, 9H, *t*-Bu), 1.89-1.94 (m, 1H, CH₂CH(NH)-), 1.96-2.03 (m, 1H, CH₂CH(NH)-), 2.31-2.37 (m, 2H, CH₂CO₂-*t*-Bu), 3.96-4.05 (m, 1H, CH(NH)-), 5.30 (s, 2H, CH₂Ph), 7.15 (d, 2H, J = 8.46 Hz, H_{arom3+5}), 7.49 (d, 2H, H_{arom2+6}), 7.83 (d, 2H, J = 9.17 Hz, H_{arom3+5}), 8.12 (d, 1H, J=7.83 Hz, NH-Ph); 8.31 (d, 2H, H_{arom2+6}); MS, (FAB), (574, 50); m/z : 597 (M⁺+23, 12), 575 (M⁺+1, 4), 519 (M - *t*-Bu - 1, 10), 463 (M - 2*t*-Bu - 2, 4);

diallyl, [4(4'-nitrophenyloxycarbonyl)oxybenzyl]-oxycarbonyl-L-glutamate (15b)

Starting from 14b (0.4 g, 1.06 mmol), 15b was obtained by a similar procedure. Purification yielded an oil (0.448g, 75.5%). ¹H-NMR, δ_H : 1.80-2.01 (m, 1H, CH(NH)CH₂-), 2.03-2.09 (m, 1H, CH(NH)CH₂-), 2.50-2.55 (m, 2H, CH₂CO₂A), 4.16-4.24 (m, 1H, CH(NH)-), 4.59 (d, 2H, J=5.29 Hz, CH₂O allyl), 4.63 (d, 2H, CH₂O allyl), 5.19-5.38 (m, 4H, CH₂= allyl), 5.31 (s, 2H, PhCH₂), 5.90-5.98 (m, 2H, CH= allyl), 7.16 (d, 2H, J=8.43 Hz, H_{arom3+5}), 7.50 (d, 2H, H_{arom2+6}), 7.58 (d, 2H, H_{arom3+5}), 8.11 (d, 2H, H_{arom2+6}); MS, (FAB), (542, 50); m/z: 563(M⁺+23, 10), accurate mass: calcd. (C₂₆H₂₆N₂O₁₁Na), 565.1434; found 565.1430.

CLAIMS

1. A compound of the formula (I):



wherein X and Y are independently chlorine, bromine, iodine, a mesyl group CH_3SO_2 or a tosyl group $\text{OSO}_2\text{phenyl}$ (wherein phenyl is optionally substituted by 1, 2, 3, 4 or 5 substituents independently selected from C_{1-4} alkyl, halogen, cyano or nitro;

n is an integer from 0 to 4 and each group R^1 , which may be the same or different when n is greater than 1, is fluorine, chlorine, bromine, iodine, hydroxy, mercapto, amino, nitro, C_{1-4} alkyl optionally containing one double bond or one triple bond, C_{1-4} alkoxy, $-\text{CONR}^7\text{R}^8$ (wherein R^7 and R^8 are as defined below), $-\text{NH}(\text{C}_{1-4}\text{-alkyl})$, $-\text{N}(\text{C}_{1-4}\text{-alkyl})_2$ or C_{2-5} alkanoyl; or when n is 2 or more and two groups R^1 are on adjacent positions on the phenylene ring, 2 adjacent R^1 groups together represent

a) C_4 alkylene optionally having one double bond;

b) C_3 alkylene; or

c) $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-$, $-\text{CH}=\text{CH}-\text{CH}_2-$ or $-\text{CH}_2-\text{CH}=\text{CH}-$ each optionally substituted with 1, 2, 3 or 4 substituents said substituents each independently selected from the group consisting of C_{1-4} alkyl, C_{1-4} alkoxy, halogen, cyano, nitro, C_{2-5} alkanoyl and $-\text{CONR}^7\text{R}^8$ (wherein R^7 and R^8 are as defined below);

m is an integer from 0 to 4 and R^2 , which may be the same or different when m is greater than 1, is fluorine, chlorine, bromine, iodine, hydroxy, mercapto, amino, nitro, C_{1-4} alkyl optionally containing one double bond or one triple bond, C_{1-4} alkoxy, $-\text{CONR}^7\text{R}^8$ (wherein R^7 and R^8 are as defined below), $-\text{NH}(\text{C}_{1-4}\text{-alkyl})$, $-\text{N}(\text{C}_{1-4}\text{-alkyl})_2$ and C_{2-5} alkanoyl; or when n is 2 or more and two groups R^1 are on adjacent positions on the phenylene ring, 2 adjacent R^1 groups together represent

- a) C4 alkylene optionally having one double bond;
- b) C3 alkylene; or
- c) $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-$, $-\text{CH}=\text{CH}-\text{CH}_2-$ or $-\text{CH}_2-\text{CH}=\text{CH}-$ each optionally substituted with 1, 2, 3 or 4 substituents said substituents each independently selected from the group consisting of C_{1-4} alkyl, C_{1-4} alkoxy, halogen, cyano, nitro, C_{2-3} alkanoyl and $-\text{CONR}^7\text{R}^8$ (wherein R^7 and R^8 are as defined below);

Z^1 and Z^2 are each independently $-\text{O}-$ or $-\text{NH}-$;

R^3 hydrogen, *t*-butyl or allyl;

Z^3 is a group $-\text{V}-\text{W}$ where V is $-\text{CH}_2-\text{T}-$ in which T is $-\text{CH}_2-$, $-\text{O}-$, $-\text{S}-$, $-(\text{SO})-$ or $-(\text{SO}_2)-$ (provided that when V has sulphur or oxygen as its second atom, W is other than $-\text{CCOH}$)

and said group V optionally further carrying one or two substituents Q^1 and/or Q^2 on carbon:

wherein Q^1 and Q^2 each independently represents C_{1-4} alkyl or

halogen; or, when Q^1 and Q^2 are bonded to adjacent carbon atoms, Q^1 and Q^2 together may additionally represent a C_3-C_4 alkylene radical optionally substituted with 1, 2, 3 or 4 substituents independently selected from the group consisting of C_{1-4} alkyl and halogen. and

W represents

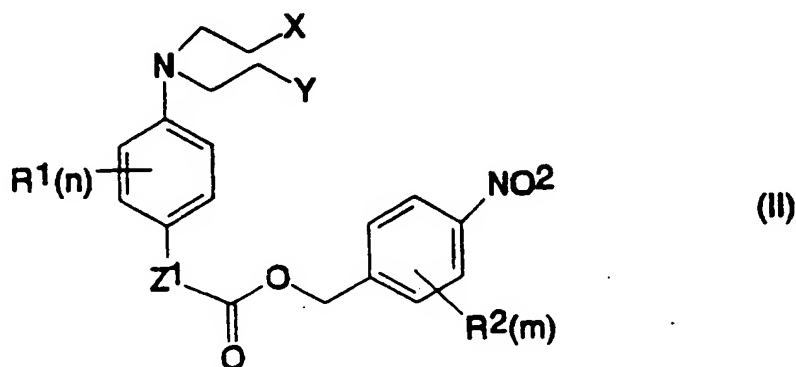
- (1) COOH .
- (2) $-(\text{C}=\text{O})-\text{O}-\text{R}^6$ wherein R^6 represents a C_{1-6} alkyl, C_{3-6} cycloalkyl or aryl (as defined in 3 below) group:
- (3) $-(\text{C}=\text{O})-\text{NR}^7\text{R}^8$ wherein R^7 and R^8 each independently represent hydrogen or a $\text{C}1-6$ alkyl, $\text{C}3-6$ cycloalkyl, aryl, heteroaryl linked to N via carbon or $\text{C}7-9$ aralkyl group wherein
 aryl is phenyl;
 heteroaryl is a 5 or 6 membered ring containing 1 to 3 heteroatoms selected from the group consisting of nitrogen and sulphur;
 the aryl moiety *per se*, the heteroaryl moiety and the aryl moiety of the aralkyl group may be substituted on carbon with 1-4 substituents selected from the group consisting of $-\text{COOH}$, $-\text{OH}$, $-\text{NH}_2$, $-\text{CH}_2-\text{NH}_2$, $-(\text{CH}_2)_{1-4}-\text{COOH}$, tetrazol-5-yl and $-\text{SO}_3\text{H}$ and the alkyl moiety may optionally carry a methyl group;
- (4) $-\text{SO}_2\text{NHR}^9$ wherein R^9 is as defined for R^7 but may additionally represent $-\text{CF}_3$, $-\text{CH}_2\text{CF}_3$ or aryl as defined above:
- (5) SO_2R^{10} in which R^{10} represents H , C_{1-6} alkyl or C_{3-6} cycloalkyl.

- (6) $\text{PO}_3\text{R}_{10}\text{R}_{10}$ (wherein the R_{10} radicals, which may be the same or different, are as herein defined)
- (7) a tetrazol-5-yl group;
- (8) $-\text{CONH}-\text{SO}_2\text{R}_{11}$ in which R_{11} represents
 - (a) C_{3-7} cycloalkyl;
 - (b) C_{1-6} -alkyl optionally substituted with substituents selected from the group consisting of aryl as defined below, C_{1-4} -alkyl, CF_3 , or halogen; and
 - (c) perfluoro- C_{1-6} alkyl; wherein
 aryl is phenyl or phenyl having 1-5 substituents wherein the substituents are selected from the group consisting of halogen, $-\text{NO}_2$, $-\text{CF}_3$, C_{1-4} alkyl, C_{1-4} alkoxy, $-\text{NH}_2$, $-\text{NHCOCH}_3$, $-\text{CONH}_2$, $-\text{OCH}_2\text{COOH}$, $-\text{NH}(\text{C}_{1-4}\text{-alkyl})$, $-\text{N}(\text{C}_{1-4}\text{-alkyl})_2$, $-\text{NHCOOC}_{1-4}\text{ alkyl}$, $-\text{OH}$, $-\text{COOH}$, $-\text{CN}$ and $-\text{COOC}_{1-4}\text{ alkyl}$; and
- (9) $-\text{M}-\text{Het}$ wherein M represents S , SO or SO_2 and Het represents a 5 or 6 membered heterocyclic aromatic ring linked to M via a carbon atom of the aromatic ring, said aromatic ring containing 1, 2, 3 or 4 heteroatoms selected from the group consisting of O , N and S said aromatic ring optionally being substituted on carbon atoms of the ring with 1, 2, 3 or 4 substituents selected from the group consisting of $-\text{OH}$, $-\text{SH}$, $-\text{CN}$, $-\text{CF}_3$, NH_2 and halogen;

and physiologically acceptable derivatives of the compound of formula (I).

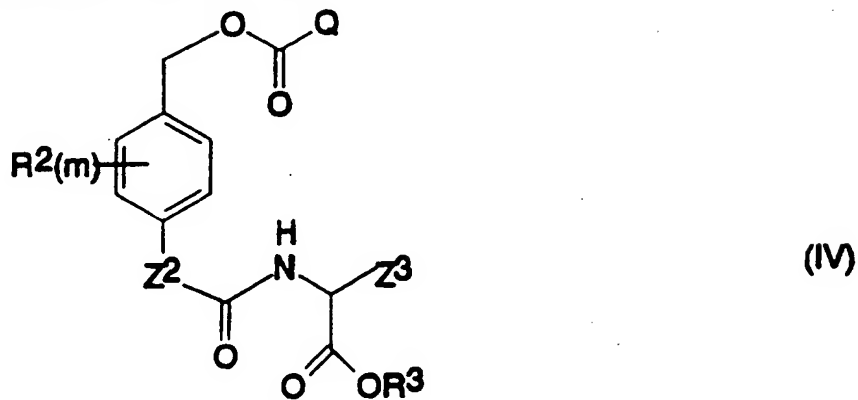
2. A compound according to claim 1 wherein R_1 and R_2 are each independently selected from hydrogen and fluorine, R_3 is hydrogen and Z^1 is a group $\text{CH}_2\text{CH}_2\text{COOH}$.

3. A compound of the formula (II):



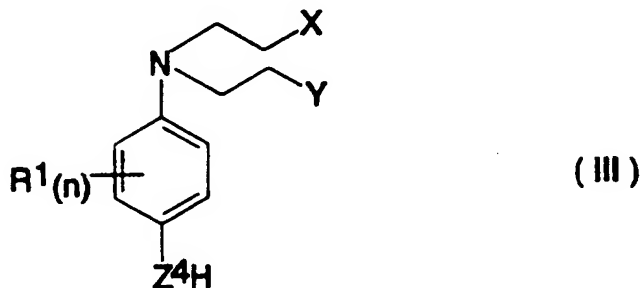
- wherein X, Y, R¹, n, Z¹, R² and m are as defined in claim 1, and physiologically acceptable derivatives thereof.
4. A compound according to claim 3 wherein R¹ and R² are each independently selected from hydrogen and fluorine.
 5. A pharmaceutical composition comprising a compound according to any one of claims 1 to 4 together with one or more pharmaceutically acceptable carriers or diluents.
 6. 4'-[N,N-bis(2-chloroethyl)amino]phenyloxycarbonyl-oxy-benzyl-4-carbamoyl-L-glutamic acid.
 7. 4'-[N,N-bis(2-chloroethyl)amino]phenyloxycarbonyl-oxy-benzyl-4-oxycarbonyl-L-glutamic.
 8. 4'-[N,N-bis(2-chloroethyl)amino]phenylcarbamoyloxy-benzyl-4-oxycarbonyl-L-glutamic acid.
 9. 4'-[N,N-bis(2-chloroethyl)amino]phenylcarbamoyloxy-benzyl-4-carbamoyl-L-glutamic acid.
 10. Use of a compound according to any one of claims 1 to 9 in a method of treatment or therapy of the human or animal body.
 11. A two component system for use in the control of neoplasia in a human or animal subject which comprises a compound according to any one of claims 1 to 9 and a vector or other delivery system capable of selectively infecting tumour cells in said subject, said vector carrying a DNA or RNA sequence encoding an enzyme capable of converting said compound to a nitrogen mustard active against said tumour.

12. A compound of the formula (IV):

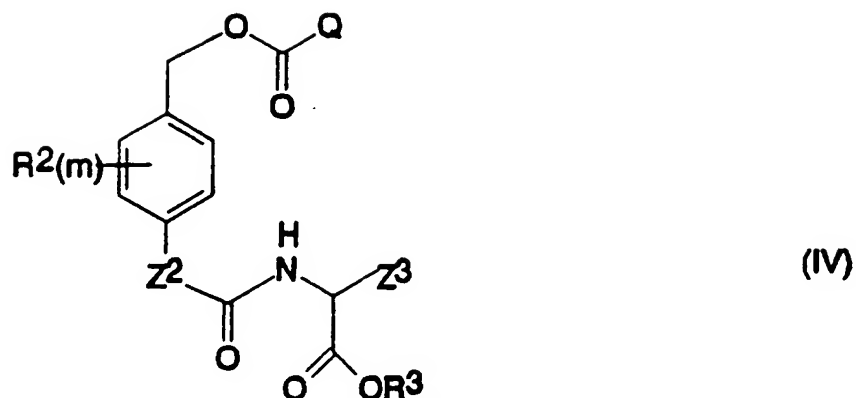


where R^2 , m , Z^2 , R^3 and Z^3 are as defined in claim 1, and Q is a leaving group, with the proviso that when R^2 is fluorine, chlorine, bromine, iodine, hydroxy, mercapto, amino, nitro, C_{1-4} alkyl, C_{1-4} haloalkyl, C_{1-4} alkoxy, C_{1-4} haloalkoxy, C_{2-4} alkenyl, C_{2-4} alkynyl, and C_{2-4} haloalkenyl, R^3 is hydrogen or a C_{1-6} alkyl group, and Z^3 is a group $-CH_2CH_2COOX^{10}$ where X^{10} is hydrogen or a $C1-6$ alkyl group, then Q is not a N-succinimidyl group.

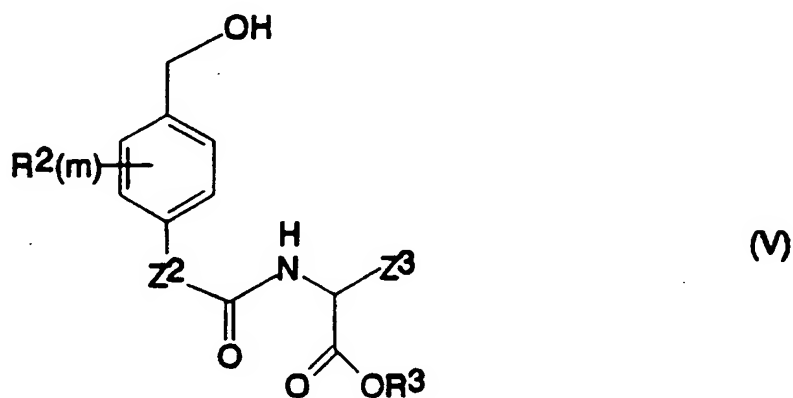
13. A process for the production of a compound of formula (I) as defined in claim 1 which comprises reacting a compound of the formula (III):



in which X , Y , R^1 and n are as defined for formula (I) and Z^4 is $-O-$ or $-NH-$ with a compound of formula (IV)



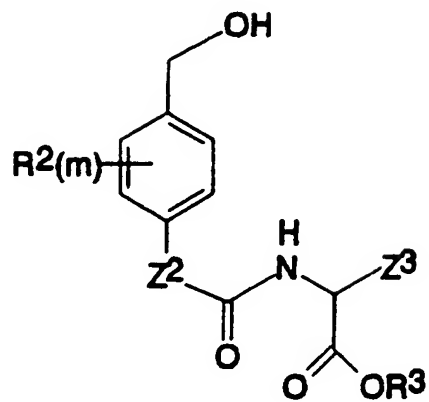
in which R^2 , m , Z^2 , Z^3 and R^3 are as defined above, and Q is a leaving group or with a compound of formula (V):



in which R^2 , m , Z^2 , Z^3 and R^3 are as defined above, and recovering the compound of formula (I) produced.

14. A process for the production of a compound of formula (II) as defined in claim 3 which comprises reacting a compound of formula (III) as defined in claim 13 with 4-nitrobenzyl chloroformate optionally substituted with from 1 to 4 group(s) R^2 as defined in claim 3, and recovering the compound of formula (II) produced.

15. A compound of formula (V)



(V)

wherein R^2 , m , Z^2 , R^3 and Z^3 are as defined in claim 1.

Fig.1.

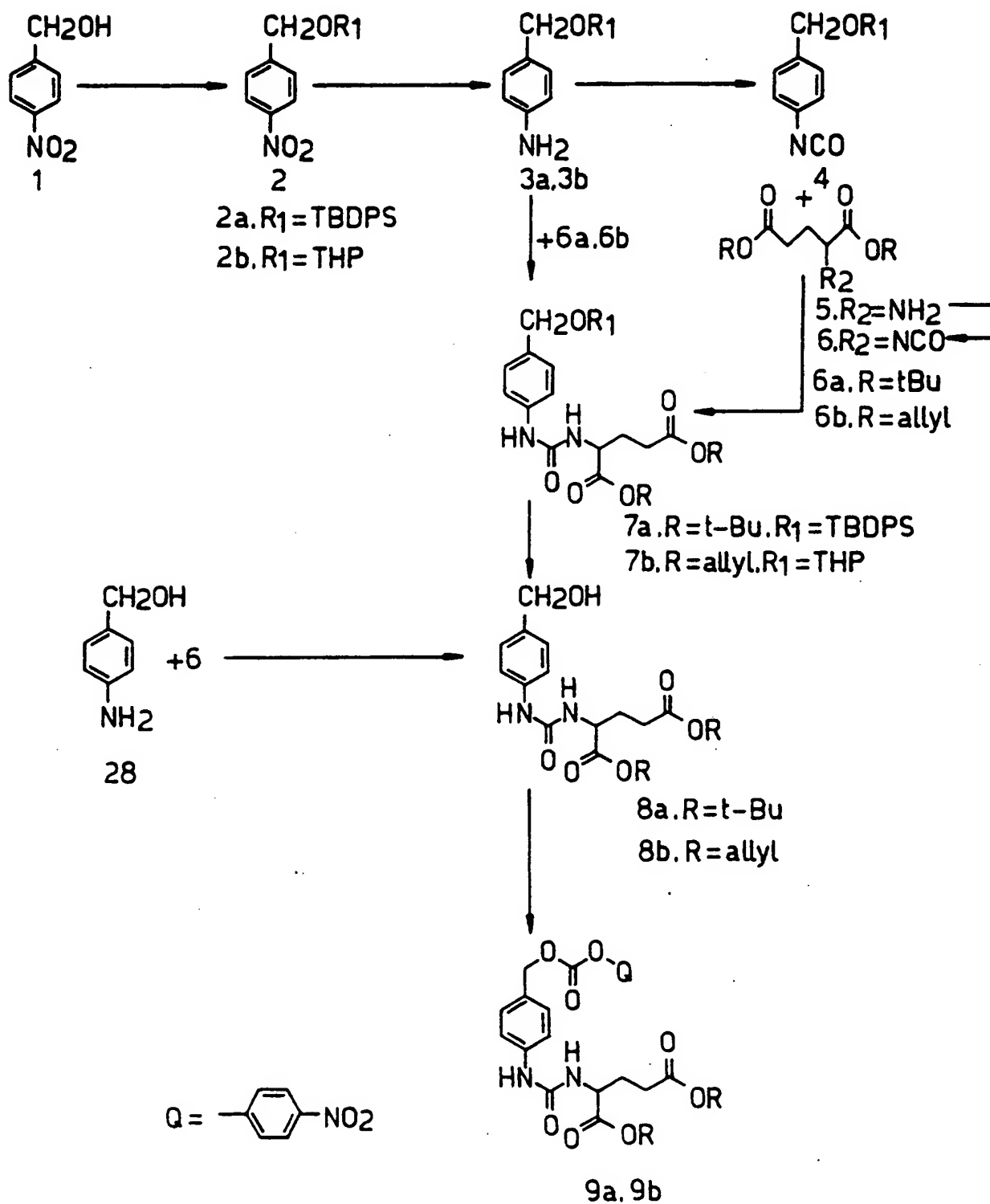
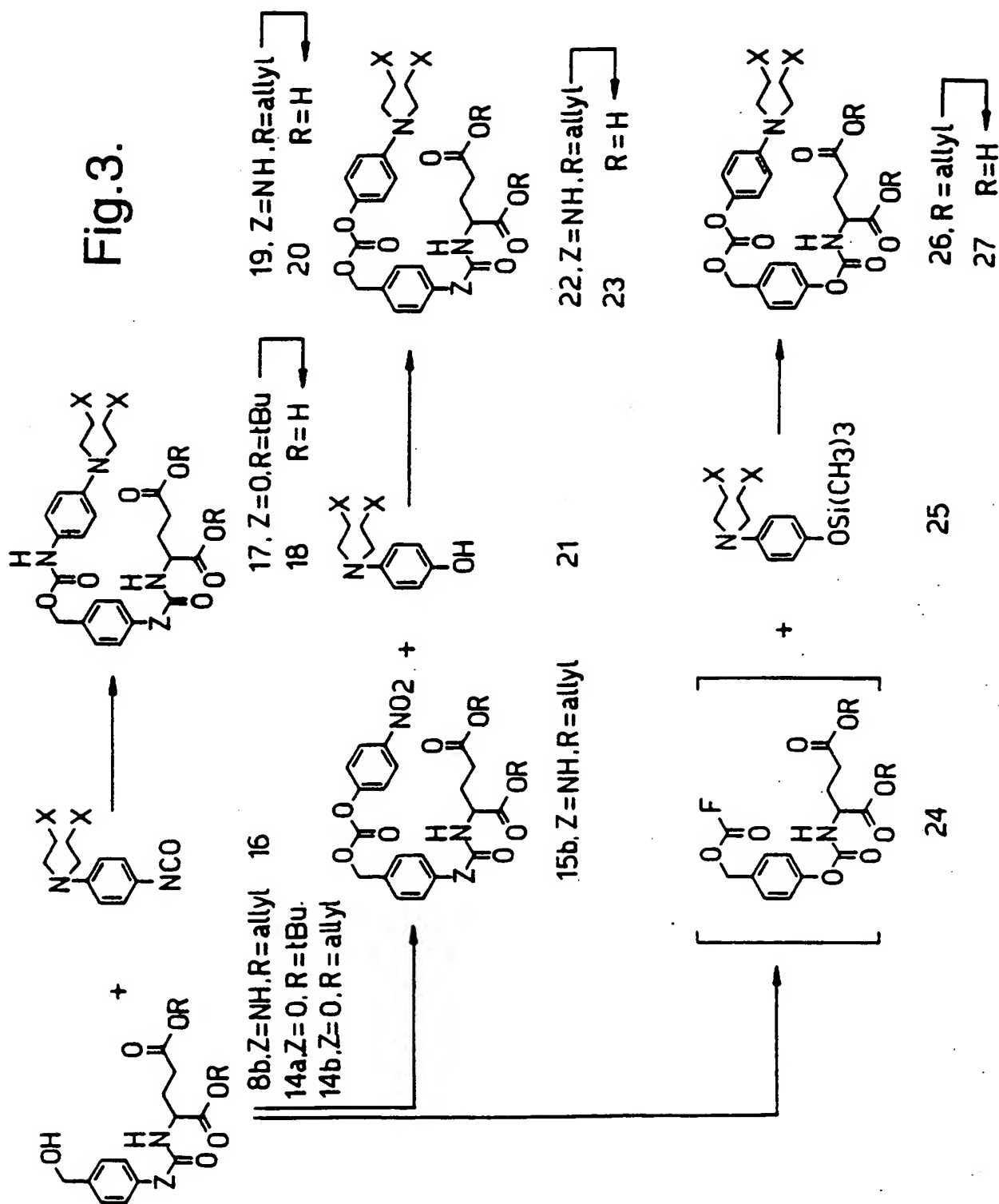




Fig.3.



According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 540 263 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD.) 5 May 1993 see claims; examples 8,9 ---	3,4,10, 11
X	JOURNAL OF MEDICINAL CHEMISTRY, vol. 37, no. 21, 14 October 1994, WASHINGTON DC, US, pages 3452-8, XP002000756 A. B. MAUGER ET. AL.: "Self-Immolative Prodrugs. Candidates for Antibody-Directed Enzyme Prodrug Therapy in Conjunction with a Nitroreductase Enzyme." see whole document ---	3,4,10, 11,14
Y	WO,A,94 02450 (ZENECA LTD) 3 February 1994 cited in the application see claims; examples ---	1,2,5-11
	-/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

17 April 1996

Date of mailing of the international search report

24.06.96

Name and mailing address of the ISA

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FR,A,1 463 764 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 21 November 1966 see whole document	1,2,5-11
Y	--- WO,A,88 07378 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD.) 6 October 1988 cited in the application see whole document -----	1,2,5-11

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		ZW-A- 9293	30-03-94

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		EP-A- 0408546	23-01-91
